RIBONUCLEIC ACID SYNTHESIS DURING IMMUNOLOGICAL PARALYSIS

THE POSSIBLE ROLE OF RIBONUCLEIC ACID AS A MEMORY ENGRAM

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Ribonucleic Acid Synthesis During Immunological Paralysis; The Possible Role of Ribonucleic Acid as a Memory Engram

by

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KEY TO ABBREVIATIONS

A - Adenine

ACA - Adenylic Acid-Cytidylic acid-Adenylic Acid

BGG - Bovine Gamma-Globulin

BSA - Bovine Serum Albumen

5 BU - 5-Bromouracil

C - Cytosine

DNA - Desoxyribose Nucleic Acid

5 FUdR - 5 Fluorouracil deoxyriboside

G - Guanine

HSA - Human Serum Albumen

IP - Intraperitoneally

6 MP - 6 Mercaptopurine

mRNA - Messenger Ribonucleic Acid

RGG - Rabbit Gamma-Globulin

RNA - Ribonucleic Acid

U - Uracil

UUU - Uridylic acid-Uridylic acid-Uridylic acid

ABSTRACT

The prevailing biological models for memory, both psychic and immunological are reviewed and evaluated, and the possible relationships between these two fields are explored. The possible role of RNA is dealt with in detail and an argument for protein as a permanent engram is put forth.

It is noted that immunological tolerance in general and immunological paralysis in particular may provide a useful tool for studying immunological memory, since with the induction of paralysis, memory of a prior sensitizing experience is abolished for a period of time. The prevailing theories of immunological tolerance are presented and evaluated in the light of existing experimental data and in the light of the data obtained by the author.

It was found that H^3-cytidine incorporation into the spleens of female CFW mice given paralyzing doses of Bovine Gamma-Globulin (BGG) after P^{32} irradiation did not differ significantly from control animals until seven days after the paralyzing dose. In the period from the seventh to the ninth day, the rate of incorporation of H^3-cytidine into the spleens of these animals dropped steadily and markedly, reaching a nadir on the ninth day of almost one-seventieth of control values.

It is concluded, therefore, that immunological paralysis results in marked inhibition of RNA synthesis in the spleens of paralyzed mice after a latent period of approximately seven days. A theory of tolerance based on complexing of antigen with DNA is presented to explain this phenomenon and is evaluated in relation to data obtained by others on tolerance.

I. INTRODUCTION - Biological Memory

Although many secret codes of foreign intrigue have been formulated and decoded by man, he has not yet deciphered the most basic and most important code(s) of all - those by which his own body records its experiences. Memory in computers is a relatively simple matter; many thousands of circuits are either open or closed. Thus, each one provides a "bit" of information, and the amount of information which can be stored in an electromagnetic memory core is given in simplified form as $(2)^{\text{number of circuits}}$ bits.

Man has approximately 10^{10} neurons in his brain, but we have not yet been able to formulate a relationship between this number and the estimated 10^{15} bits of information which he accumulates in a lifetime.[1]

If each neuron could exist in only two states (like a circuit), the number of bits of information which man should be able to retain at any one moment in time would be $[(2)10]^{10}$ which is much more than the 10^{15} bits which we usually acquire. Yet, in reality more than one neuron is needed for each piece of information, as shown by the relatively minor intellectual losses sustained when rather large areas of non-specific cortex are ablated.

There are at least three biological phenomena which require a system for storage and retrieval of information: the genetic phenomenon, the immune response, and psychic memory. Although many questions still remain unanswered, the role of nucleic acids in genetics and protein synthesis, including amino acid coding, while not yet fully elucidated is certainly known to be basic.

Questions such as degeneracy still want for solutions, but the fact that the triplet codon UUU signifies phenylalanine,[2,3] that ACA signifies aspartic acid,[2,3] etc. is established. Nucleic acids are thus without a doubt a means by which genetic information is stored. These facts noted, we shall deal no more with genetics and shall concern ourselves with those bits of information acquired postnatally. Before leaving genetics, however, we should point out that coded genetic information can at times cross the boundary into the realm of psychic information, e.g. in the case of instinctual behavior which we may consider genetically programmed and also into the realm of immunological information, e.g. in the case of natural antibodies which exist without any exposure to antigen viz. the anti-A and anti-B blood group antibodies and antibodies to certain Gram-negative organisms.*

The engram in immunologic and psychic memory is still undetermined. Many theories as to how such information is processed, stored, and retained have been formulated, and we shall now examine the most prominent of these.

Before doing so, however, let us define the term "memory" in the simplest terms possible. For our purposes, memory will be merely the capacity to contain information and the mechanism by which this information is stored. In order for us to realize its existence, however, there must also be 1) means for processing the information prior to storing it, e.g. the conversion of events into electrical impulses and perhaps these latter into chemical codes in psychic memory. 2)Storage of the final result of the above process

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* While this view has numerous adherents,[96] many investigators[183] do not believe that natural antibodies exist.

3) Some means of retrieval to provide access to the information. This, in the case of a code, would involve a deciphering mechanism. 4) Some outward specific demonstration as a result of the deciphering, e.g. antibody synthesis or a behavioral act.

It has been suggested by many that the processes involved in psychic and immunological memory may be either similar or identical. It is, for example, stated in The Chemistry of Thinking , "It may be that there is something more than a superficial similarity between the reaction of lymphocytes to the....antigen and the events which occur in a neuron following the disturbances in its milieu produced by volleys of impulses."[4]

Silverstein[1] has written an excellent review in which he compares immunological and psychic memory. The similarities are striking. Inquiring whether the similarity of terms employed in these two fields represents merely an inappropriate choice of terminology, a basic inadequacy of the language, or perhaps truly an underlying fundamental relationship, he goes on to elucidate their similarities (Fig. 1).

In both systems, a basic conversion of the raw input must be made - to a nerve impulse in the case of a sensory phenomenon and to something else (perhaps degradation products) in immunology. Benacerraf and Maurer[5] have shown certain substances will be antigenic only if they contain l-amide linkages for which digestive enzymes exist, whereas the d-amide linkage compounds are non-antigenic, the body being unable to degrade these molecules. Further evidence for this fact is that strain 2 guinea pigs can produce antibodies to portions of insulin to which strain 13 cannot, and perhaps this is due to a

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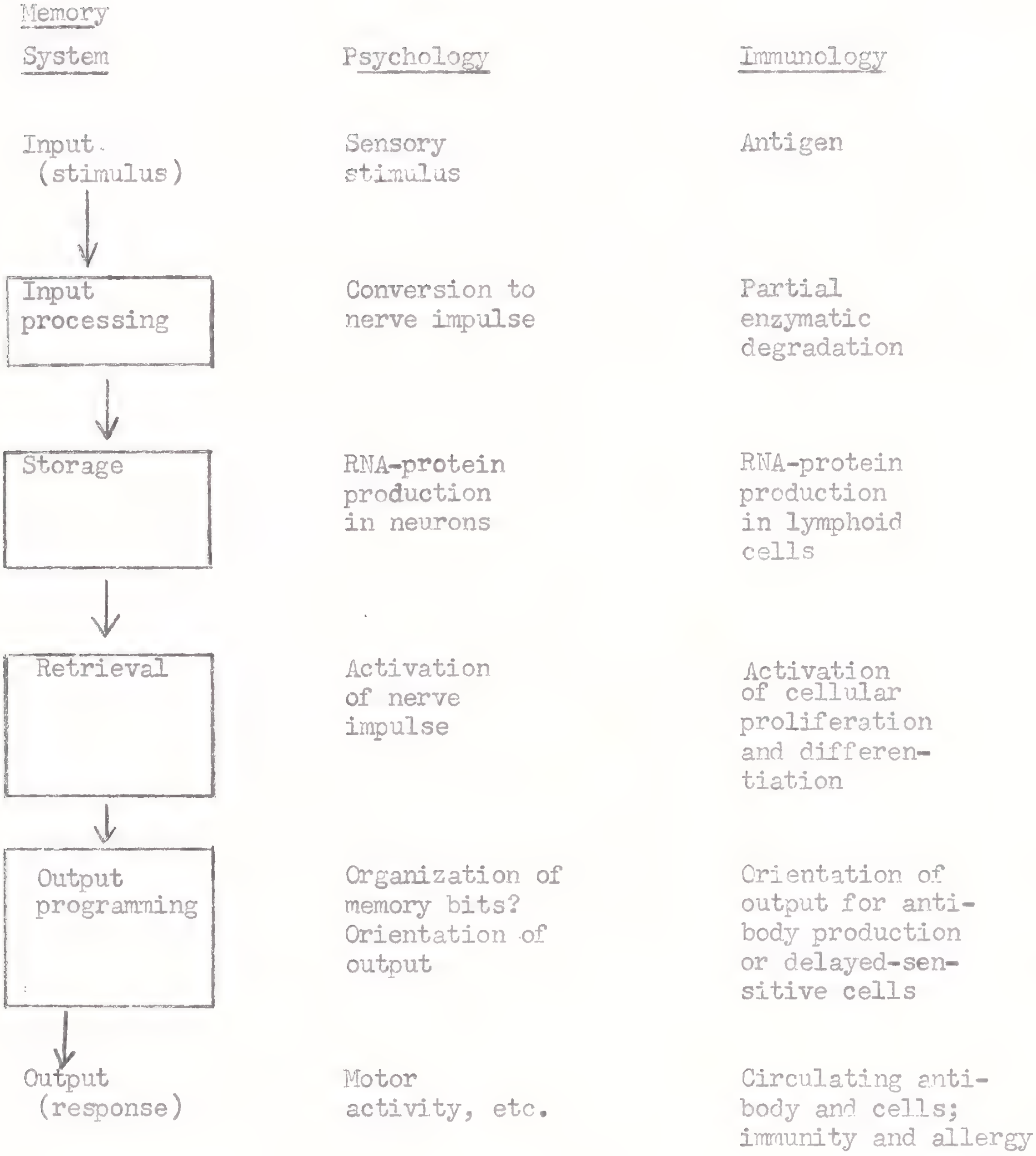

Memory System	Psychology	Immunology
Input (stimulus)	Sensory stimulus	Antigen
Input processing	Conversion to nerve impulse	Partial enzymatic degradation
Storage	RNA-protein production in neurons	RNA-protein production in lymphoid cells
Retrieval	Activation of nerve impulse	Activation of cellular proliferation and differentiation
Output programming	Organization of memory bits? Orientation of output	Orientation of output for antibody production or delayed-sensitive cells
Output (response)	Motor activity, etc.	Circulating antibody and cells; immunity and allergy

Figure 1. The functional components of the memory system, with psychologic and immunologic analogues.

Adapted from Chase, R. A. *Perspectives Biol. Med.*
by Silverstein, A. M., "Immunologic and Psychic Memory", *Neurosciences Research Program Bull.* 1, p.4 (1963)

genetic absence of an appropriate catabolic enzyme.[6] Also, the time of onset of the ability of a fetus or newborn to react to different antigens varies from antigen to antigen,[7] and it has been postulated[5] that this might be due to the possibility that all immunological capabilities mature early but the enzyme systems required for degradation of all antigens might not appear simultaneously.

That RNA plays a role in both the psychic and immunological memory systems can be seen by the specific detrimental effects on both of anti-nucleic acid drugs which will be discussed in detail later. For example, both have been claimed to depend on DNA-dependent RNA,[8,9] and as will be discussed later, claims have been made for passive transfer of both types of memory with "educated" RNA. Lawrence[10] has, however, effected long term transfers of immunological memory in men with cell free extracts of leukocytes containing what he terms "transfer factor", a low molecular-weight substance of unknown composition, but known to be neither DNA nor RNA.

RNA synthesis and protein synthesis increase in both neurons and lymphocytes as a result of their respective types of stimulation. Repetition improves both processes (anamnestic response in immunology, reinforcement in psychology) and the phenomenom of generalization of location with passage of time is common to both. Thus, for example, hippocampal and temporal cortex ablation will abolish recently learned information, but after several days the engram somehow becomes generalized over the cortex. Similarly, shortly after immunization, excision of the lymph node draining the site of immunization will abolish the immune response while this does not occur after a few hours.

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The mechanism of the latter, we believe, we understand, viz. the transport via the blood and lymph of cells and antigen; that of the former remains fertile ground for speculation.

Both psychic and immunological memory have aspects which may be considered long and short term. If, for example, an animal is shocked after each trial, it fails to learn, but if one waits between one and four hours after each trial, then shocks the animal, it will learn normally. This is the period, presumably, during which the information is converted from electrical impulses to more stable form. The period for this information to become spread and generalized in the cortex is, however, as will be seen later from the experiments of Flexner, considerably longer (3-6 days).

In immunology, the first response is that of a macroglobulin (19S) antibody. The production of this antibody lasts only so long as the antigen persists. If sufficient antigen has been given, approximately one week later, 7S antibodies begin to be formed. These are the antibodies associated with the anamnestic response. If just 19S antibodies are formed, antibody production with the second challenge is no different from the first. The relative amounts of 19S and 7S are a function of many things, especially the nature of the antigen.[1,11-14]

A further analogy between the two systems is that of stimulus generalization i.e. cross reactions occur with similar antigens, the maximal response occurring to the actual antigen, and similarly Pavlov showed that an animal conditioned to react to one wave-length of light will respond to others, the response decreasing on either side of the original wave-length.

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The effects of X-ray, 8-azaguanine and other anti-metabolites on both responses are similar and extremely important to this discussion and will, accordingly, be discussed in greater detail later in this paper.

In summary, there are a great many similarities between immunological and psychic memory, enough to have made Hechter and Halkerston postulate an antigen-antibody mechanism for psychic memory,[15] and enough to make one wonder whether the same biological principle might underlie the functions of both. Nevertheless, with the present dearth of knowledge in these fields, any conclusions about a possible relationship between them would, as Silverstein concludes[1], be premature.

We might note here that while Hechter and Halkerson would explain psychological memory in terms of immunology, much has been written in the Russian literature to try to explain immunology in terms of conditioned reflexes involving the nervous system.[16-19] They have noted some changes, they claim, in antibody titer after stimulation of various regions of the brain[20] and have studied the effects of neuroses[21] and schizophrenia[22] on the immune response. Since much of this work has not yet been confirmed and is, in fact, not believed by many on our side of the iron curtain, we will not go any further with it except to point out that this is just one more attempt to link these two phenomena which require information storage.

We will therefore progress directly to an examination of the major theories which have been proposed to explain information

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II. Process vs Chemical Memory

Memory may be stored for long periods in one of two general ways. The first is by something going on continuously, much as a moving flywheel conveys the information that it was started turning. Its position at any instant is unimportant with respect to conveying this fact. On the other hand, that same flywheel if moved from its usual location in three dimentional space (as opposed to spinning while standing in one place) would convey yet another type of information: that it had been moved. This is analogous to a structural code for memory, while the spinning flywheel is analogous to a process theory.

III. Process Theories of Memory

The process theory which has received the most attention is the neuron loop theory, viz. neuron A excites neuron B which then, in turn, excites neuron C, etc. until the last neuron in the series re-excites neuron A, and thus the series is able to continue indefinitely. This would require, of course, the loop to be long enough such that enough time would elapse before coming back to neuron A so that this neuron would have recovered from its refractory period.

Another recent popular idea along similar lines is that of a gene which can exist in several states, but which in whatever state it finds itself, produces its own inducer such that the gene produces inducer which thereby allows it to produce more inducer and maintain its current state.[23]

These and other process theories of memory, however, are not widely held because physiological processes such as protein synthesis

and brain waves can be stopped by treatment with cold, shock, drugs, and hibernation without loss of long term psychic or immunological memory.[9,24] Long term psychic memory is also not lost when the brain is sliced in many directions[25] which would have cut many loops, thus again showing memory to be generalized, a phenomenon which could be accounted for by many loops, but is more likely due to structural changes in many neurons.

Morowitz[26] has shown that information to specify a living system can survive cooling to within two degrees of absolute zero. When he cooled Artemia eggs to this temperature and left them for one week, they hatched normally, thus showing the information in this case is structural, not process.

IV. Structural Theories of Memory

Far more popular than the process theories are the structural and especially the chemical theories of memory. DNA, RNA, proteins, and lipids have each been postulated as engrams of memory.[27-32]

Of these compounds, the nucleic acids have aroused the most interest. Norbert Weiner has written:

> "It is becoming abundantly clear that the nucleic acid complexes not only play a fundamental role in genetic memory, but that they probably play an analogous role in nervous memory ... and we shall have to consider the interplay of what Professor Francis Schmitt of MIT calls "dry" neurophysiology, dealing with the established nervous network, and "wet" physiology, which is going to center more and more about the nucleic acids."[33]

The investigator whose work has had the most profound effects on this field is Holger Hydén of Göteborg, Sweden. Hydén and Egyházi[9] found that right-handed rats taught to become left-handed had

increased nuclear RNA and base ratio changes (increased purine to pyrimidine ratio) in the fifth and sixth cortical layers on the right, the left side serving as a control.

In a more telling experiment Hydén and Egyházi[34] taught rats to climb a wire set at 45° from the horizontal in order to get food. Not only was the nuclear RNA of the Deiter's (vestibular) cells increased from an average of 650 micrograms to 730 micrograms per cell, but the base ratios were significantly altered. The adenine to uracil (A/U) ratio went from a control value of 1.06± .08 to 1.35 ± .10. Hydén, in recent publications[35,9,36] notes that such RNA increases and alterations in base ratios were observable in nuclei of both neuronal and glial cells, while in an earlier publication[37] he noted a 5% increase in neuronal RNA and a 30% decrease in glial RNA.

Hydén believes the increased RNA which he has shown to be nuclear is chromosomal RNA. One problem, however, with the whole idea of RNA as a memory molecule is the existence of six types of cellular RNA,[30] namely chromosomal RNA, nucleoplasmic RNA, two types in the nucleolus (one of which is messenger RNA), soluble RNA (also called transfer RNA), and ribosomal RNA.

We might note also that brain RNA increases from the age of three to the age of forty, stays constant from forty to sixty, and then falls. Certainly a person continues to increase his store of knowledge between the ages of forty and sixty and he does not forget fast enough for this to free RNA for coding. We are thus faced with three possibilities on this point. First, either large amounts of RNA are non-functional and only acquire function by rearrangement of their base sequence. Second, perhaps RNA is only necessary transiently

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for the learning process, and some other molecule (e.g. protein as suggested by Hydén) is the long term engram, and thirdly, that RNA is not involved at all. Our knowledge at this time does not permit us to come to any conclusion but we may note that protein and nucleic acid synthesis rates in the brain are among the highest in the body - even higher than in the pancreas.[37]

Hydén postulates that a given frequency pattern of impulses would affect glial RNA first, and that this would be followed by transfer of nucleotides from glia to neurons to cause release of repressed regions of chromosomal DNA leading to production of DNA-dependent RNA which would then serve as a template for specific proteins.[9] These proteins would remain as a permanent engram and dissociate to a substance which activates the transmitter substance when they are activated by the same pattern of frequencies which led to their synthesis. Hydén mentions that a frequency of 500 cycles per second is equivalent to 2×10^{-18}W ($E=h\nu$). This might be enough energy to dissociate a proton.

Hydén thus assumes in this paper that all knowledge is in DNA, not RNA, but that proteins and not RNA serve as an active file for information. (The RNA changes disappeared in his experiments twenty hours after they were first noted.[9]) In analogy to theories of antibody formation, this is a selective theory rather than an instructive one.

Although Hydén's theory as described above from this publication[9] seems to be of the "selective" type, he formerly interpreted his data[34,31] as being in favor of an "instructive" approach in which he pictured the sensory stimulus as inducing a new and stable sequence of nucleotides directly in RNA which then, in turn, determined the structure of specific proteins.

Morrell[38] criticized Hydén's earlier instructive theory on the grounds that it cannot explain how an electric current can induce a molecular rearrangement which would thereafter be immune to further electrical currents. He thus interprets the same type of data in favor of a selective approach in which all possible engrams are genetically determined in the DNA. The stimulus is postulated by Morrell to activate selectively a given DNA-RNA sequence (much as Hydén has most recently postulated) and, realizing the speed of interaction which is required and that this is not ordinarily associated with reactions involving macromolecules, he has proposed that charge-transfer reactions (which are very rapid reactions) might play a role.

In some earlier work, Morrell[39] produced an epileptic lesion on the cortex using an ethyl chloride spray. Soon afterward, he found a "mirror" focus in the contralateral cortex which also showed paroxysmal epileptiform discharge and which was self-sustaining, but which disappeared when this cortex was undermined. It could, however, be elicited again by stimulating the surrounding normal cortex, e.g. with metrazol. This was interpreted by Morrell as showing that the "learned" behavior of the secondary lesion had been "remembered" even after months of inactivity. He then showed that the nerve cells within the area of the mirror focus showed increased stainability with pyronin, which stains RNA, (using a methyl green-pyronin stain), and he therefore concluded that changes in RNA had occurred in these cells secondary to continuous synaptic bombardment.

While the increased staining is interesting, it has been claimed by Hydén[9] that staining with methyl green-pyronin in cell sections is not specific for qualitative changes in RNA. Morrell, however, feels that this does represent increased RNA and has interpreted it as already described.

Learning studies in planaria, small flat worms, have aroused great

interest among those concerned with the mechanism of memory. These animals can be trained in various ways (light-shock conditioning, mazes, etc.). It has been found that if "educated" worms are cut in half and ribonuclease is added to the media in which the heads and tails are regenerating, it blocks retention of conditioning in animals regenerated from tails but not in those regenerated from heads.[40] "Education" in these animals has been transferred by cannibalistic ingestion of educated planarians by naive worms.[41] That this transfer of "education" was due to RNA transfer was shown by Zelman et al[42] who considerably decreased the total number of trials necessary to educate naive planaria by injecting them with RNA from conditioned planaria.

Recently, Egyházi, Hydén, and John[43] showed that the base ratios of A+U/G+C in RNA from planarian head ganglia were much higher in animals conditioned with light and cathodal shock than in untreated controls and animals treated with light and anodal shock, but similar changes were seen with random light and cathodal shock, therefore implying that the changes in base ratios and in total RNA were not associated with the conditioning per se, but were due to the stimulation.

We might point out here that while there is general agreement regarding the increased rate of RNA synthesis following neuronal stimulation, there remains great confusion regarding the role of base ratio changes in the brain. Hydén, for example, before the above experiment, claimed that the total amount of RNA may go up with randomly increased activity, but without a change in base ratios, the latter occurring only in specific learning. In support of this theory, he had found in earlier studies that increased neuronal stimulation, sensory or motor, increases the neuronal content of RNA, protein, and enzymes, without any change in base ratios.[9] On the other hand, Geiger[44] demonstrated a change in RNA composition in cortical neurons after only thirty seconds of

stimulation which contradicts Hydén's hypothesis that these changes do not occur secondary to increased activity, as do Hyden's own recent findings with planaria.

While great confusion exists regarding base ratio changes, there can be little question of the fact that RNA plays some role in both immunological and psychic memory. In addition to the work of Hydén and Morrell which we discussed, many other bits of evidence have strongly implied a role for RNA in memory.

Yeast RNA on chronic administration to old men has been claimed by Cameron et al[45-48] to improve memory in cases of arteriosclerotic senility. Cook et al[49] found enhanced response to a shock motivated response with greater resistance to extinction in rats given 160mg/kg./day of yeast RNA intraperitoneally.

Dingman and Sporn[50] showed that 8-azaguanine (a purine analogue which causes formation of a non-functional RNA) injected intracisternally (132 micrograms) impairs rats' ability to learn a new maze without affecting their ability to traverse a previously learned maze. This same purine analogue has been shown by Chamberlain et al[51] to prolong the interval required for "fixation of experience" in a study employing rat spinal cord. These authors also found that tricyanoaminopropene (a compound which increases RNA synthesis in neurons and glia) improved performance of animals in avoidance conditioning.

All of the evidence which we have listed so far for RNA involvement in memory has concerned psychological memory. Its role in immunological memory is certainly as prominent and, perhaps, somewhat better demonstrated. In fact, Fishman[52-54] has shown that RNA extracted from macrophages exposed in a micro diffusion chamber to antigen, when incubated with lymphocytes will lead to production

of antibody against the antigen, thus certainly showing that RNA is capable (at least for a short time) of carrying information regarding antibody specificity.

Similarly, Mannick and Egdahl[55] found that "neutral" lymph node cells from non-grafted rabbits were altered to a state of transplantation immunity when incubated with RNA extracted from lymph nodes of rabbits receiving skin homografts. This was shown by a skin reaction on injection of these cells into the donor of the skin homografts. Similarly, Šterzl and Hrubesová[56] transferred antibody formation to non-immune rabbits using spleen nucleoprotein from immunized rabbits. Further studies of this type have been carried out in Japan by Noro[57] and Konda et al[58]. These experiments, like those of Fishman, however, while implying a role for RNA in memory, are not conclusive since it is possible that all that is being transferred is the RNA which controls the synthesis of antibody protein, because as we recall, the mechanism of protein synthesis is believed to consist of the formation of messenger RNA (mRNA) on and complimentary to DNA, attachment of amino acids to acceptor RNA (also called transfer or soluble RNA) which in turn attaches in the proper places to the specific codon of the mRNA which is now attached to ribosomal RNA.

We may further look with interest at the contrast between the experiments of Fishman and those of Mannick and Egdahl and of Šterzl and Hrubesová. Fishman used macrophage RNA, Mannick and Egdahl used RNA from lymph node homogenates, presumably therefore from both lymphocytes and macrophages, and Šterzl and Hrubesová used RNA from spleen, again presumably from both macrophages and lymphocytes. The experiments of Fishman re-raise the question of

of antibody against the antigen, but certainly a [illegible] than [illegible] capable (at least for a short time) of carrying information regarding antibody specificity.

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We may further look with interest at the [illegible] experiments of Fishman and those of [illegible] and Hrubesova. Fishman used macrophages [illegible] used RNA from lymph node homogenate, presumably [illegible] both lymphocytes and macrophages, and Sterzl and [illegible] RNA from spleen, again presumably from both [illegible] lymphocytes. The experiments of [illegible]

the necessity of partial digestion of antigen[5], and one can only wonder whether the RNA transferred to the lymphocytes from the macrophages is mRNA for synthesis of antibody protein by the latter, or perhaps, it is the engram from which the lymphocyte gains its "knowledge" of the antigen and then makes its antibody - either retaining this engram or making a new one from it.

Much as RNA transferred from cells making antibody can induce antibody synthesis in non-immune cells, RNA from penicillinase producing strains of B. cereus has been shown capable of inducing penicillinase production in non-penicillinase producing strains.[59] The same objections regarding protein synthesis are, however, applicable here.

As noted earlier, Dingman and Sporn[50] showed that 8-azaguanine can inhibit learning of a new maze in mice. Similarly, this and other anti-nucleic acid drugs inhibit the immune response,[60-62] and to complete the analogy, the primary response is much more significatly affected than is the secondary since presumably it is during the primary that the knowledge of exposure to antigen is coded. Doses of 3 mg./kg./day and 6 mg./kg./day of 6 mercaptopurine[63-65] which significantly inhibit the primary response in rabbits had almost no effect on the secondary response. It has been shown, however, that these drugs can sometimes inhibit the anamnestic response also, e. g. if rabbit spleen cells are removed after secondary stimulation, they will incormporate C^{14}-amino acids into synthesized antibody, a process which is inhibited by 5-bromouracil (5BU) and 5-fluorouracil deoxyriboside (5-FUdR).[66] La Plante et al[67] and Condie et al[68] have shown that 6MP in larger doses can completely block the secondary response to BSA.

In analyzing these and other experiments with similar data, Hitchings and Elion[62] postulate that the difference between the effect of 6 MP on the primary response and that on the secondary is quantitative rather than qualitative. We would like to speculate further, however, that the reason for this difference may be that in one case (the lower dosage i.e. in the primary response) coding of information regarding antibody and antigen configuration is being impaired, while in the case of the higher dosage, (necessary to block the secondary response) RNA synthesis necessary for antibody protein synthesis is being disturbed non-specifically. Schwartz and Dameshek[69] have also shown, as have others, that 6 MP administered with antigen will tend to induce tolerance to that antigen, a topic to which we shall return later.

Species differences in the effects of 6 MP[70], 8-azaguanine,[60] and other anti-metabolites on antibody formation have been reported, and while it is usually used for the reverse,[71] occasionally 6 MP can accel~~l~~erate a graft vs host reaction.[72] The whole subject of chemical suppression of the immune response has been extensively reviewed by Hitchings and Elion.[62]

Radiation, like anti-metabolites, if properly timed[73] can inhibit the immune response, having most of its effect on the primary response and very much less on the secondary.[74,75] Radiation and anti-nucleic acid compounds work synergistically to inhibit immune responses as shown by the fact that while 900R in dogs was insufficient to permit successful bone marrow homografts, small doses of 6 MP prior to irradiation were followed by successful marrow transplantation.[76]

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Also, as is well known, unresponsiveness in adult animals can be induced by antigen administration with either radiation or anti-nucleic acid compounds, thus further implying but by no means proving the similar mechanism of action of these two approaches.

The purpose of all of the foregoing was to give some impression of the weighty but certainly not conclusive evidence marshalled behind the nucleic acids as the engramatic molecules of memory, both psychic and immunological.

We have, up to this point, discussed only RNA as a possible engram but DNA, like RNA, is coded from four different basic units, adenine, thymine (instead of uracil), guanine, and cytosine. Thus DNA, as has been pointed out with reference to genetics, is capable of coding information. It is possible that the changes which occur in neurological and immune memory take place in DNA, and that the observed changes in RNA are only secondary to primary changes in DNA, the RNA being synthesized on the DNA and complimentary to it in the usual manner. While this is, of course, possible,[30] the basic beliefs regarding the greater mutability of RNA and the greater stability of DNA would mitigate against a change in the base composition of cellular DNA. It has been suggested,[30] however, that the DNA in the nervous system may be more mutable than DNA elsewhere, and we would like to point out that it seems to us that this would be reasonable since the DNA in the mature nervous system does not have to fulfill the function of DNA in the rest of the cells of the body - that of carrying information for replicating the cell.

Some evidence exists, in fact, that DNA may be more important than RNA in the immune response. Simić et al[77] found that when the

immune response is blocked with 5-bromouracil, this effect can be counteracted by thymine but not by uracil. Similarly, Dutton[66] found that antibody formation inhibited by 5-FUdR was enhanced by thymine and not uracil. This is especially interesting since thymine is made from uracil by methylation, and the results are therefore unexplainable at present.

While all of the above presumes a change in the DNA i.e. an instructional theory, we must keep in mind the possibility of a selective theory, in which, for example, the information could be stored in DNA and only "released" by other compounds or removal of other compounds e.g. histones from histone-DNA complexes, thus not requiring a change in DNA base composition. Also, Burnet's clonal selection theory which will be discussed later and whereby information of exposure is postulated to be stored by the multiplication of appropriate cells, may be looked at as a "magnification" of knowledge already in the DNA of the cell, again not requiring any change in this DNA's composition. We might note here, too, that many of the data cited in support of the role of RNA e.g. effects of radiation and purine and pyrimidine analogues apply as well to DNA.

Hydén's theory, as we recall, also implies a role for proteins in addition to that postulated for nucleic acids. Further support for the role of proteins in memory comes from the work of Flexner et al[78] who injected puromycin (which inhibits protein synthesis) bilaterally into the hippocampi and temporal cortices of mice and found that this caused loss of short term intellectual memory. After 3 to 6 days, the engram spread such that it was necessary to inject most of the rest of the cortex to cause loss of memory. Reversal learning was lost while

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original learning (which had become longer term memory) was retained after bilateral injection into the hippocampi and temporal regions. Since puromycin inhibits protein synthesis, as noted, these experiments imply a role for protein in learning but, as is evident from the comparative paucity of discussion, relatively less work has been done with respect to the investigation of proteins as molecules which code information as compared with the nucleic acids.

We should like to point out at this time, however, that if we consider a protein code, one thing becomes immediately apparent: the extreme increase in efficiency of proteins as information stores over nucleic acids. If we assume the number of "common" amino acids to be 21, then a 3 unit code or "3 letter word" would contain 21^3 bits as opposed to a "3 letter word" of the nucleic acid code which contains 4^3 or 64 bits. This huge increase in efficiency would require much less protein synthesis to code a given amount of information than the amount of nucleic acid synthesis required to code the same information.

We might compare this decrease in necessary space to code a given number of bits of information to the efficiency of our own decimal system as opposed to the binary system. In the binary system, the number 21 (in the decimal system) would be written 10101, while it takes only two digits in a system based on ten. As we recall, the units digit is the number of single units; the digit to the left of that is to be multiplied by the base to the first power - ten in the decimal system, two in the binary system, and the number to the left of that is to be multiplied by the base squared, etc. Thus, 21 equals $1 \times 1 + 2 \times 10 + 0 \times 10^2$, etc. In the binary system, as written above, we have $1 \times 1 + 0 \times 2 + 1 \times 2^2 + 0 \times 2^3 + 1 \times 2^4 = 21$ (in decimal system)

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In sum, then, proteins would provide a more concise code than would nucleic acids.

With regard to the question of lipids, almost no experiments were carried out to investigate the question of whether these molecules serve as memory molecules.

To summarize, we must recall that no molecule, protein, nucleic acid, or lipid has been shown to satisfy criteria which would prove it to be the engram beyond a reasonable doubt. The following criteria have been suggested by Dingman and Sporn[27]:

1) It must undergo a change of state in response to the experience to be remembered.
2) The altered state must persist as long as the memory can be demonstrated.
3) Specific destruction of the altered state must result in permanent loss of the memory.

They point out that the observed changes in RNA may be transient (as did Hydén), and therefore RNA would not be the permanent engram. In any case, further research is needed.

Other theories of memory, both psychic and immunological have been postulated e.g. that of Hechter and Halkerston[15] which proposes a system whereby the cell body produces antibody-like protein which at junctional surfaces recognizes specific amine-histone complexes produced by other neurons and corresponding to the informational code. This antigen-antibody reaction at the cell surface facilitates neuronal firing. Learning, it was suggested, exists when the specific antibodies in key neurons exceed a certain level. These levels, it is postulated, decrease with time unless reinforcing stimuli are repeated. According to Hechter and Halkerston altered RNA is the engram, for it directs the synthesis of the antibody protein.

We wonder, however, why, if the RNA remains constant, as they claim, would the antibody level drop with time.

Hechter and Halkerston propose methylation of bases resulting in a change in base pairing as the mechanism of alteration of the RNA. They have, however, performed no experiments to confirm this.

While it has been taken for granted for quite some time that antibody production although initiated in the presence of antigen, "is carried on long after all antigen has disappeared," [79,80] some recent work by Speirs[81] implies that perhaps micro-quantities of retained antigen serve as the means by which memory of a former antigenic experience is established and maintained. He found small quantities of tritiated antigen passed from macrophage to macrophage, and many of these antigen-laden macrophages came to the site of injection after a second injection of antigen.

This theory receives support from the work of Garvey[82] which showed that S^{35} BSA remained in the liver for long periods, .02% of the injected amount remaining 140 days after injection. This was, they calculated, 10^{14} molecules. They noted that the BSA appeared bound to a salt-soluble ribonucleic acid fraction, and found, as did Vredevoe and Nelson[83] that this retained material was more antigenic than the pure material. Rittenberg and Nelson[84] proposed that digestion of antigen within macrophages was a necessary step leading to information which was "likely to be contained in a nucleoprotein" and which was capable of directing antibody synthesis. This would be consistent with the work of Benacerraf et al[5] and of Fishman[52-54] which we discussed earlier. The question of the role, if any, played by retained antigen still remains an enigma, not only with respect to the anamnestic response but also with respect to immunological tolerance in which

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connection it will be dealt with later in this paper.

The oldest structural theory of psychic memory is that of Cajal[85] and others[86] who postulated that learning involved changes in synaptic relations between neurons and the establishment of new connections by means of axonal and dendritic growth. This would be consistent with a necessity for increased synthesis of both RNA and protein, and has, accordingly, been espoused recently by several people[86] with only relatively minor modifications.

Having discussed the major theories of information storage, both psychic and immunological, we will henceforward limit discussion to the question of how immunologic memory is stored with particular emphasis on the mechanism of immunological tolerance, paralysis, or unresponsiveness.

In order to discuss this properly, however, we must first consider the theories put forward to explain the synthesis of antibody.

V. Theories of Antibody Synthesis

Volumes have been filled with papers on theories of antibody synthesis. As there is a very recent excellent review of these,[87] we will concern ourselves here with just a skeletal outline of the prevailing theories and a short discussion of how they relate to immune paralysis and immunological unresponsiveness in general.

The theories may first of all be divided into "instructive" and "selective" categories, the former implying that the antigen directs the synthesis of antibody de novo, the latter implying that the antigen merely selects its antibodies from among the many proteins, the information for whose synthesis the body already has in its DNA.

The methods proposed by which either instruction or selection is carried out are varied. Thus, for example, instructive theories can be further subdivided by what is instructed. Is the antibody directly synthesized on the antigen (thus direct template synthesis),[88-91] or does the antigen instruct another molecule,[92] protein,[93,94] DNA,[95] RNA, or other, which either directly or indirectly controls synthesis of antibody (indirect template hypothesis).

Various methods for selection have been proposed. Jerne[96] proposed that "natural antibodies" of many types exist, and then the antigen selects its antibodies, the complex then being phagocytized, the antibodies split off and then replicated. The main objection to such a theory[87] is, however, that it would have protein control its own synthesis which is contrary to our present theory of protein synthesis.

Another type of selective theory is analogous to the regulator-operon theory of Jacob and Monod.[97] In one theory of this type,[98] the antigen is postulated to combine with repressor, thus allowing RNA synthesis on the operon. This mRNA codes the composition of the antibody. We thus have a cell capable of producing antibody to the specific antigen. The cell would then replicate according to Burnet's clonal selection theory,[99-102] which will be discussed shortly. While the regulator-operon theory is here used as a possible explanation for antibody synthesis, others[103] have claimed that it and enzyme induction may be more analogous to immune tolerance than to antibody formation.

The most famous selection theory of all and the one which has had the greatest impact on immunology is that espoused by Burnet and Lederberg.[99-102,104] It assumes that each cell produces only one

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Another type of selective theory is analogous to the repressor-operon theory of Jacob and Monod.[illegible] In one theory of this type, the antigen is postulated to combine with a repressor, thus allowing RNA synthesis of the operon. This would code the composition of the antibody. We thus have a cell capable of producing antibody to one specific antigen. The cell would then replicate according to the clonal selection theory, [illegible] which will be discussed shortly. While the repressor-operon theory is often used as a possible explanation for antibody synthesis, [illegible] have claimed that it and other induction may be more analogous to immune tolerance than [illegible]

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type of antibody, and therefore selection of a single cell for proliferation results in increased synthesis of a specific type of antibody. The importance of cellular proliferation for antibody synthesis has been documented,[105-107] and the fact that single cells do indeed produce only one antibody has been supported by the work of Nossal and Lederberg,[108-111] Coons,[112] and White.[113] There is, however, some evidence on the other side of the fence. Attardi, Cohn, Horibata and Lennox[114] found that 2% of single cells did produce two types of antibody against unrelated phage, and Cohn and Lennox[115] further showed using unrelated phage types, that rather than having mutual exclusion between the synthesis of one antibody and another, "If any given cell makes antibody to T_2, it apparently has a greater probability of also making antibody to T_5." Also, Trentin and Fahlberg[116] showed that if a single spleen cell is allowed to propagate and repopulate a lethally irradiated animal, that animal will be able to react to as many as four antigens.

Such data provides strong arguments against the clonal selection theory. Lederberg[104] postulates, however, that new stem cells are constantly arising with new potentialities. While this might explain the eventual reactivity to four antigens in the repopulated animal, and while it may well be true, it has also been used by Lederberg[104,117] to explain the necessity for persistent antigen in maintaining immune tolerance, a purpose for which, as will be seen later, it is not sufficient. (In fact, the necessity for persistent antigen has not been proved.)

The above point excluded, though, one of the major considerations in favor of the clonal selection theory is the simple explanation it

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provides for tolerance. In the words of Fischer, "While the selective theories may meet the problem of immunological tolerance with less than a fully satisfactory explanation, the instructive theories were generally formulated before the question gained prominence and they failed to confront it at all."[87]

VI. Immunological Tolerance

With this introduction to tolerance, we will now examine this phenomenon in more detail. The beginnings of the current concepts of tolerance date back not quite two decades when, in 1945, Owen[118] described chimerism in twin cattle. These twins both carried two types of red blood corpuscles, accepted skin grafts from each other and rejected grafts from other animals normally.[119,120] These observations led Burnet and Fenner[99] to postulate that the recognition of "self" and "not-self" is determined early in life and therefore not genetically determined. This concept was proved correct by Billingham, Brent and Medawar[121,122] when they injected fetal strain A mice with lymphoid cells of CBA mice. These cells were accepted, and thereafter the mice accepted all grafts from the donors of the cells. This experiment and variations of it were repeated many times by many investigators. All discovered the same thing: The organism's first non-genetically acquired information is what is "self" and what is "not-self." Our question is how this information is stored, but before making this inquiry, let us ask how the organism manifests this knowledge.

The normal organism makes antibodies, gamma-globulin protein molecules, to combine with most foreign materials of large molecular size, especially proteins. Yet, except in rare instances, it does

not make antibodies to its own proteins. Nevertheless, there are several conditions in which the organism will not make antibodies even to foreign proteins. We call this a state of immunological unresponsiveness. Several excellent reviews have been written on this subject.[103,123-125]

Medawar[103] divided unresponsiveness into five categories:

1) Tolerance after exposure very early in life (reviewed by Brent and Medawar[126]

2) Non-reactivity after exposure to high doses of radiation. (as described by Dixon and Maurer[127])

3) Sulzberger-Chase phenomenon[128-132] in which ability to form antibody to certain chemicals is abolished by oral or systemic administration of the antigen before giving the immunizing dose.

4) Immunological paralysis - First described by Felton[133] in 1949, this is a specific unresponsiveness which he produced using large doses of pneumococcal polysaccharides. In this category we would also like to include the tolerance induced to foreign red blood corpuscles after a huge innoculum,[134] and to large grafts.[135-137] With respect to the latter, we might note here that each antigenic group of the antigen must be "tolerated." Hence, in split tolerance, one antigenic determinant of a molecule may be tolerated while antibody may be formed to another. Therefore, it is easier to induce tolerance to a protein or graft which differs little in histocompatibility genes from the host.[138]

5) Protein overloading paralysis - This is a specific unresponsiveness induced in adult animals by administering a very high dose of soluble protein antigen.[127,133,139] It is important that these proteins be soluble and without adjuvant since often the

same protein if precipitated with adjuvant will prove antigenic.[140] As noted by Chutná and Hraba,[141] HSA precipitated with antibody in doses which would produce tolerance is not capable of inducing tolerance but instead produces an immune response, and Smith[142] showed that BSA-antibody precipitate is not capable of producing tolerance, but if tolerance already exists, it can prolong it.

As both (4) and (5) are specific for the compound in excess (this has been questioned,[143] and will be dealt with later), most authors consider them as one phenomenon, as we shall do here.

Since the time of Medawar's review, a new class of unresponsiveness has been added which we would group with (2), viz. unresponsiveness after administration of antigen with anti-metabolites, especially 6MP.[62, 69,145,146] For example, Mc Laren[145] has shown that animals given spleen cells while being treated with 6MP would subsequently show permanent tolerance to skin grafts from the donors of the spleen cells. In fact, as already noted,[76] anti-metabolites have been shown to be synergistic with radiation. Nevertheless, some investigators have not had such good luck in inducing tolerance by these methods,[147] but it has been suggested by some that their dosage may have been too low. Most others feel, however,[62] that 6MP administered with antigen in adults is not as potent an inducer of tolerance as is perinatal injections of antigen, although it has been claimed that the depletion of cells by radiation and anti-metabolites might return the animal to a fetal-like state with subsequent great proliferation of antibody-synthesizing cells.

While there are some differences between the various types of unresponsiveness (as described by Medawar[103] and Smith[123]) many believe that immunological tolerance induced by the various methods

are merely different manifestations of the same phenomenon,[148-151] and Hitchings and Ellion[62] state, "Thus immunological tolerance merges into immunological paralysis." Dresser[140] similarly feels that his results, "suggest that there is no difference between states of immunological unresponsiveness induced in neonatal and three month old adult mice." He states that others' difficulties lie in failure to centrifuge their protein, since in adults, small amounts of particulate matter may serve as adjuvant. Simonsen[148] believes that tolerance acquired neonatally and paralysis are identical except for the larger amount of antigen required for immunological paralysis.

It was originally felt that immunological paralysis was different from tolerance, because the lack of antibody in paralyzed animals was thought to be due to removal of antibody by the excess antigen.[152,153] This was shown not to be so by the failure of paralyzed cells to produce antibody when transplanted to irradiated hosts.[154-156] Also, Secarz and Coons[151] failed to demonstrate antibody in the cells of paralyzed animals by using fluorescent anti-gamma-globulin. Lastly, Dixon and Maurer[127] showed that the last stages of antibody removal in paralyzed animals are by non-immune mechanisms. Thus, it has been shown that immunological paralysis, like immunological tolerance, is a central failure of antibody production.

For purposes of discussion, therefore, we shall consider tolerance which is produced by radio-mimetic drugs, radiation, excess antigen in untreated animals, etc., as various aspects

of a single phenomenon. Whether the phenomenon which accompanies protein overloading should be referred to as immunological tolerance, immunological paralysis, or immunological unresponsivensss is a matter of preference, as noted by Dresser[140] who presents an entire discussion on the terminology of the unresponsive state and chooses the word "paralysis" for unresponsiveness in the presence of excess antigen. We have used and will continue to use this term for protein overloading unresponsiveness, but will also use the term "tolerance" for this phenomenon. The term "tolerance" will also be used as the general term for the phenomenon of unresponsiveness to antigenic stimuli .

Since we are concerned with the informational aspect of tolerance, we will note that it is considered as specific as the immune response. Thus, an animal somehow codes the information that it is not to make antibody to a specific molecule, while retaining its capacity to synthesize antibody to other, unrelated antigens.[127,69,151] Schwartz and Dameshek[69] showed, for example, that animals tolerant to BGG would react to HSA administered simultaneously, and Secarz and Coons[151] demonstrated that immunological tolerance to BSA did not interfere with antibody formation to diphtheria toxoid. Dixon and Maurer[127] claim that it was specific with respect to cross-reacting antigens, in contrast to Smith[142] who points out that, as in antibody formation, there is some partial tolerance between cross-reacting antigens. In diametric opposition to this, Liacopolos, Halpern and Perrament[143] point out that guinea pigs given sensitizing amounts of rabbit gamma-globulin (RGG) or

ovalbumen during the stage when paralysis is being maintained by bovine serum albumine (BSA) are not tolerant to the RGG if tested before the tenth day of unresponsiveness, but after the fifteenth day (if BSA injections are continued) the unresponsiveness "spreads" to include the RGG and other compounds. This non-specific unresponsiveness, they claim, ceases about eight days after cessation of BSA injections while tolerance to BSA continues. This would imply that immunological paralysis is a specific phenomenon "early" and "late" in its course.

After a great deal of time has elapsed even the specific tolerance lapses. The reaction of the animal to a challenge with antigen after tolerance has lapsed is a source of much disagreement. We mentioned the work of Dixon and Maurer[127] earlier, from which it appears that when a paralytic dose of antigen is slowly eliminated down to an amount which would be antigenic, the animal does not become immunized (since the last bit of antigen is eliminated logarithmically). We therefore expect a primary response to a subsequent injection of antigen as was found by Smith and Bridges[157] whose BSA-tolerant rabbits after lapsing of the tolerant state reacted to a BSA challenge with a primary response rather than a secondary.

Characteristic of the confusion and contradictions that reign in this field is the data of Siskind[158] which show that animals who have lost tolerance react with a secondary (anamnestic) response on re-challenge, and not with a primary. It is thus not possible at this time to come to any conclusions regarding the animal's future immunological reaction to an antigen to which it

swellmen during the stay when [illegible] using [illegible] bovine serum albumin (BSA) are not tolerant [illegible] before the tenth day of unresponsiveness, but after the fifteenth day (if the injections are continued) the unresponsive or "tolerant" st[illegible] includes the [illegible] other components. This non-reactive[illegible] ness, they claim, ceases about eight days after cessation of the injections while tolerance [illegible] continued. [illegible] that immunological paralysis is [illegible] the [illegible] "[illegible]" [illegible] "[illegible]" in its course.

After a great deal of work has [illegible] been [illegible] the tolerance topic, the [illegible] antigen after tolerance has lapsed is a source of [illegible]. He mentions the work of Dixon and Maurer[17] [illegible] stress that when a paralytic dose of antigen is given [illegible] come to an amount which would be [illegible] become immunized since [illegible] of antigen [illegible] lose gradually. We [illegible] a [illegible] subsequent injection of antigen [illegible] since BSA-tolerant rabbits [illegible] respond to a new challenge with [illegible] normally.

[illegible] of the [illegible] continued [illegible] in this field is the [illegible] of [illegible][1] [illegible] who [illegible] BSA-tolerant [illegible] to [illegible] [illegible] (unimmunized) [illegible]

has been made tolerant after that tolerance has lapsed. The fact that tolerance does lapse, however, is interesting. One of the explanations put forward for this is a requirement for persistence of antigen to maintain tolerance.

Much more attention has been paid to the role of persistent antigen as a means of coding the information required for immunological tolerance, and especially for immunological paralysis, than has been paid to the role of retained antigen in the immune response. It is not yet, however, known whether the persistence of antigen is, indeed, necessary for retention of specific immunological unresponsiveness. On the other hand, it is known that the amount of antigen necessary to induce tolerance in an animal is a function of the antigen itself,[151,124] of the form[140] and route[128,132] by which it is given, and of the animal which is used. There is a strong correlation between the length of time for which paralysis persists and the dose of antigen used to induce it.[134,157,159,160] Similar host differences exist with respect to unresponsiveness which is produced by employing radio-mimetic drugs in rabbits vs guinea pigs,[70] and in mice vs rats.[60]

There is much evidence for the view that antigen is necessary for the maintainance of the tolerant state.[123,127,134,157] It has, for example, been shown by Dresser and Mitchison[134] that tolerance in chickens to foreign red blood corpuscles required these for its persistence. Using a Cr^{51} tracer, they found that twenty-five days after elimination of the last detectible amount of antigen, the animals would react with an immune response if challenged with antigen. Smith[123] concludes that their data "demonstrate concisely

the requirement for antigen to sustain the tolerant state."

While many hold the above view, just as many hold the opposite view.[132,151,155] Dixon, in the discussion of the work of Dresser and Mitchison,[134] pointed out that if an animal is made unresponsive by a large amount of soluble protein and then passively given antibody to eliminate all circulating antigen, tolerance still remains. This, admittedly, does not rule out the possibility of microscopic amounts of antigen persisting at critical sites, but it certainly does rule out the necessity for the large amounts of antigen which were required by Dresser's system. Deitrich and Weigle[155] also conclude that since tolerance remained after transfer of paralyzed cells to lethally irradiated recipients, persistence of antigen is probably not necessary for maintaining the tolerant state. Let us recall once more, however, that the amount of antigen necessary for induction of paralysis will vary widely depending on the animal and the antigen, and perhaps microquantities intracellularly will suffice for some antigens.

In summary, we can only agree with Hašek et al[124] in noting that the requirement for antigen in maintaining the tolerant state is still undecided, but as they point out, the question is an important one since the necessity of persistent antigen for maintenance of tolerance would strongly favor a theory of blockage of some function rather than elimination of cells or cellular structures as Burnet and others have postulated.

If one were to hold a subcellular selective theory of antibody formation, e.g. that an antigen induces antibody formation by

the requirement for antigen to sustain the tolerant state."

While many hold the above view, just as many hold the opposite view.[132,133,[illegible]] Dixon, in the discussion of the work of [illegible] and Mitchison[134] pointed out that if an animal is made [illegible] by a large amount of soluble protein [illegible] then [illegible] given [illegible] to eliminate all circulating antigen, tolerance [illegible]. This, admittedly, does not rule out the persistence of [illegible] of antigen persisting at cellular level, but it certainly does rule out the necessity for the large amount of [illegible] required by Dresser's hypothesis. [illegible] and [illegible] has argued that since tolerance remained after transfer of [illegible] lethally irradiated recipients, persistence of [illegible] not necessary for maintaining the tolerant state. [illegible] some, however, that the amount of antigen necessary [illegible] of paralysis will vary widely depending on the animal and [illegible], and perhaps microquantities intracellularly will [illegible]

In summary, we can only agree with [illegible] et al[1??] [illegible] that the requirement for antigen in maintaining the [illegible] is still undecided, but as they point out, the question [illegible] one since the necessity of persistent antigen [illegible] tolerance would certainly favor a theory of blockage of some [illegible] rather than elimination of cells or cellular [illegible] and [illegible] are possible.

If one were to hold a [illegible] formation, [illegible]

dissociating histone-DNA complexes, thus allowing RNA to be formed on that DNA and antibody protein to be formed on the RNA, then a consistent theory of tolerance would be, we believe, that in tolerance these histone-DNA complexes are somehow maintained and strenthened, perhaps with a large amount of antigen forming a more stable ternary complex of histone-DNA-antigen.

Such a theory would, of course, require continued presence of antigen, especially in the nucleus. The finding of twice as much S^{35} labelled antigen in the nucleus of tolerant animals[123] as compared with controls, and the selective concentration of antigen in liver nuclei in tolerant rabbits[161] are consistent with our theory as is the data of Smith[142] regarding the increased permeability to antigen (BSA) in newborn rabbits. This would provide a possible explanation why with mature animals larger doses of antigen are required to produce tolerance, since a larger concentration gradient is required to overcome the permeability barrier in order to achieve the proper concentration at the place where the proposed complexes are formed.

Since this theory has the antigen combining with DNA, it would predict an inhibition of RNA synthesis since a portion of the DNA is "tied up" and RNA could not, therefore be synthesized upon it. A theory of antigen complexing with RNA would not necessitate decreased RNA synthesis, although it would impair antibody (protein) synthesis.

Smith[123] has proposed a theory of this type whereby antigen is postulated to combine with mRNA. He claims that this combination

dissociating dislocation-boundary, the dislocations [illegible] form on that and are entirely [illegible] to be formed on the [illegible], the consistent theory of [illegible] by, nevertheless, that in coherence these [illegible] the surface [illegible] considered, perhaps [illegible] a more stable energy configuration of dislocation-boundary.

Surface energy which, of course, requires consideration of surfaces, especially in the [illegible]. The [illegible] such[12] [illegible] surfaces in the [illegible] of [illegible] in common with [illegible], and the [illegible] [illegible] our theory [illegible] of [illegible][12] [illegible] [illegible] to [illegible] in [illegible]. This [illegible] provides a possible [illegible] why [illegible] of surface [illegible] when a large [illegible] tension gradient is required [illegible] in order to [illegible] process consistent [illegible] forms.

These [illegible] for [illegible] it would predict an [illegible] of [illegible] of the line [illegible] and [illegible] [illegible]. A theory of [illegible] [illegible] [illegible].

[illegible][123] has [illegible]

"might inhibit rather than stimulate proliferation and differentiation."

Of the remaining theories of tolerance, Burnet and Lederberg's postulation which we alluded to earlier, namely that tolerance to an antigen is created by the elimination of the clone which is competent to make antibody to the antigen is the most popular. Such a mechanism in its unrefined form would be in disagreement with a requirement for persistent antigen, but as we pointed out earlier, Lederberg[104] postulates constant mutation of stem cells in the adult animal, some of which, by chance, may make antibody to the antigen. Therefore, excess antigen would be necessary to destroy these cells as they appeared. Smith and Bridges[157] calculated that the amount of antigen necessary to maintain tolerance was 10^{10} molecules, but as we noted earlier the entire question of the necessity of antigen is still unanswered.

It has been shown that whole body radiation (600R)[162-165] is capable of breaking down tolerance. Several hypotheses are set forth by Nossal[162] as possible explanations of this data: 1) That certain cells are immunized when antigen is given and the rest are rendered tolerant, and that perhaps the immunized cells are more radio-resistant, thus giving them an advantage in post-radiation proliferation. 2) That radiation induces regeneration of cells or sub-cellular entities originally destroyed by the antigen. 3) That radiation induces mutation which allows emergence of new clones (which is the explanation Burnet and Lederberg favor), and 4) We would add as a possibility, although we do not favor it, that perhaps the radiation breaks up a code molecule which has coded the fact that

"might inhibit rather than [illegible]

Of the remaining theories of tolerance, [illegible] postulation which we alluded to earlier, namely that tolerance to an antigen is created by the elimination of [illegible] competent to make antibody [illegible]. Such a mechanism in its simplest form would [illegible] a requirement for continuous antigen, but as we [illegible] Lederberg[14] postulates constant mutation [illegible] animal, some of which, by chance, may make antibody [illegible] therefore, except antigen would be [illegible] as they appear. "Natural antigens"[15] [illegible] of antigen necessary [illegible] as we noted earlier the entire question of the necessity of antigen is still unresolved.

It has been shown that [illegible] capable of breaking down tolerance. [illegible] forward by Nossal[16] as [illegible] certain cells are [illegible] [illegible] radio-resistant, [illegible] proliferation. [illegible] sub-cellular structure originally [illegible] radiation induces mutation [illegible] which is the explanation [illegible] could act as a restriction [illegible] the reputation [illegible]

the animal is tolerant, possibly nucleic acids or protein (nucleic acids being especially sensitive to radiation). We would add, lastly, what we believe to be the most reasonable explanation, viz. 5) that radiation destroys cells, thereby making available nucleic acid precursors which somehow break tolerance, as will be discussed later. This would be in agreement with the fact that nucleic acid precursors enhance antibody formation[144] as does radiation at the proper time,[166] presumably by the same mechanism (although other mechanisms have been postulated). Radiation can, as is well known inhibit the immune response, and as we have noted, help in the induction of tolerance.

With respect to radiation induced tolerance, we would like to point out Smith's[123] postulation that the interference of radiation with antibody synthesis is probably brought about, "through disruption of active DNA synthesis," since the incorporation of P^{32} into DNA ceases in heavily irradiated animals and antibody synthesis in immune animals which has been inhibited by radiation can be restored by partially depolymerized nucleic acids (but not by purines, pyrimidines, nucleosides, or nucleotides) as demonstrated by Taliaferro and Jaroslow.[167]

In support of our interpretation of the mechanism of how radiation breaks down tolerance is the work of Feldman et al[146] who made rats tolerant to human serum albumen (HSA) both by using X-irradiation and 6 MP. They then were able to reactivate the immune response in all of these by injection of either spleen nuclei or nuclei treated with nucleases. It was also noted that the spleens of the treated animals incorporated 3.2 times more

the animal is tolerant, possibly nucleic acids or protein [illegible] acids being especially sensitive to radiation. We would say, lastly, what we believe to be the most reasonable explanation, i.e., that radiation destroys cells, thereby making available nucleic acid precursors which somehow break tolerance, as will be discussed later. This would be in agreement with the [illegible] that nucleic acid precursors enhance antibody formation[124] [illegible] at the proper time,[160] presumably by the same mechanism [illegible] other mechanisms have been postulated. Radiation may, as is well known, inhibit the immune response, [illegible] we have noted, help in the induction of tolerance.

With respect to radiation induced tolerance, we [illegible] to point out results[123] postulating that [illegible] radiation with antibody synthesis is probably [illegible] "[illegible] disruption of active DNA synthesis," since [illegible] into DNA occurs in heavily [illegible] in immune animals which has been inhibited by [illegible] restored by partially depolymerized nucleic acids [illegible] pyrimidines, nucleosides, or nucleotides [illegible] Ballisteros and Dansler.[illegible]

In support of our interpretation of the mechanism of [illegible] radiation breaks down tolerance is the work of [illegible] [illegible] made rats tolerant to human serum albumin [illegible] [illegible] and [illegible]. They were able to restore the immune response in all of these by injection of [illegible] [illegible] of nucleic [illegible] [illegible] of the [illegible]

thymidine eighty hours after radiation than spleens of irradiated animals that had not received nuclei. These data, as the authors point out, can hardly be reconciled with the concept of Lederberg[104] that reactivation of the non-functional immune response is brought about by a random mutational process. They point out that it seems unlikely that administration of DNA or its degradation products could lead to a surge of mutations. They conclude, therefore, that drug and radiation induced tolerance is not secondary to "killing off" cells but is a result of intracellular damage "possibly at the level of information....for the production of specific antibody." Whether the intracellular lesion is destruction or elimination of a structure, or merely blocking of a function by antigen is debatable, and as we have noted earlier, the former might not require persistence of antigen for maintainance of tolerance while the latter certainly would.

One can speculate that it is possible for radiation to impair some permeability barriers, thereby enabling antibody in smaller quantities to gain access to places where DNA-histone-antigen complexes may be formed. It would follow then that tolerance could be overcome by large quantities of nucleic acid degradation products which by virtue of their increased concentration would be able to competitively displace antigen from the DNA where its presence was preventing synthesis of new DNA (on the DNA) and new RNA (on the DNA), as we proposed earlier.

There has been much speculation by others also as to which intracellular sites might be affected in tolerance, and it has been

postulated by Hašek et al[124] that antigen can attack one of two sites, one leading to antibody formation, the other to inhibition. Others postulate that a high concentration of antigen in the cytoplasm around the nucleus leads to inhibition or destruction of some subcellular apparatus (as described in Hašek's paper[124]), but we can, at present, say nothing certain as regards the intracellular activity of the antigen. Nevertheless, in our opinion, it seems far less reasonable to ascribe a specific function to the cytoplasm around the nucleus than to assume that inhibition occurs by some blockage of the conventional DNA-RNA-protein pathway of antibody synthesis (e.g. DNA-histone-antigen complexes), and as noted earlier, there is some evidence for this (e.g. the increased amount of antigen in the nuclei of tolerant cells[123,161]).

The results of Feldman,[146] which we have noted earlier, are not only in agreement with our theory but also are consistent with our data. In addition, they represent the only measurement of immunocyte nucleic acid metabolism during the tolerant state except for our own study, and they note that thymidine incoporation into DNA was greatly depressed in tolerant cells.

If indeed, however, we are to adhere to a chemical theory of tolerance, it seems curious that the thymus appears to be necessary for breakdown of the unresponsive state. It has been reported[168-170] to be necessary for recovery of the immune response after whole body irradiation and, as has been shown by Clamen and Talmadge,[171] thymectomy in an adult tolerant to a specific antigen prevented reappearance of reactivity with respect to that antigen. Perhaps, then

correlated by means [illegible] has [illegible] of the [illegible] projection of [illegible] constant [illegible] about the nuclear [illegible] cellular [illegible] structures, they [illegible] of the [illegible] nucleus [illegible] of the [illegible] e.g., [illegible] are [illegible]

The results [illegible]

[illegible]

there is a thymic factor which must "de-repress" cells which have been made tolerant (e.g. by helping to break up DNA-histone-complexes, if such exist).

The remaining theories of tolerance (besides the various aspects of antigen retention, cellular elimination, and elimination or blocking of sub-cellular mechanisms, which we have already discussed) include the analogy with enzyme induction in bacteria, namely that an antigen in large quantity may induce enzymes which destroy it so fast that antibody cannot be formed, or in an animal tolerant from birth, catabolic enzymes may be retained. Such a theory is unlikely when one considers that persistence of antigen in tolerant animals is longer than in immune animals.

Lastly, the theory that "immunologically incompetant lymphocytes" are produced and result in tolerance has been put forth without any experimental support by Gorman and Chandler.[172] They postulate that these proliferate in response to the antigen and thus compete with the immunologically competent cells. The evidence against such a theory is overwhelming. First, while antibody synthesis can be explained by the presence of a few competent cells, tolerance requires every competent cell in the body to be unresponsive to the antigen. It would then be necessary for the proliferation of these cells to compete successfully with every scattered cell capable of making antibody to this antigen. Furthermore, our data and that of Feldman et al[146] argue strongly for a decrease in cellular metabolism and proliferation, not an increase, as this theory requires.

Having reviewed the theories of immunological tolerance,

there is a larger [illegible] that "degeneration" will [illegible] made tolerant [illegible] to [illegible] than [illegible].

The remaining theories of [illegible] of [illegible], and [illegible] sub-[illegible], which we have already [illegible] analogy with enzyme induction in bacteria, namely [illegible] large quantity may induce enzymes [illegible] addition cannot be [illegible], or in an [illegible] substrate may be [illegible]. [illegible] the [illegible] that [illegible] of [illegible] former [illegible] in [illegible].

[illegible] the [illegible] in [illegible] experimental support by [illegible] these [illegible] in response to [illegible] and that [illegible] commonly [illegible] the [illegible] of [illegible] even [illegible] it is [illegible] with [illegible] in [illegible] for [illegible] [illegible], not an [illegible].

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we will now go on to our own data which, we believe, supports the hypothesis that tolerance results not in increased cellular proliferation, but in a marked decrease in RNA synthesis as previously noted, perhaps due to antigen complexing with DNA.

VII. The Experiments - Background

The study of immunological tolerance in order to gain further insight into the mechanism of the immune response and immunological memory may be considered to be analogous to the classical approach of extirpation of an organ or inhibition of an enzyme in order to learn something about its mechanism of action. To learn which changes occur during a state of non-function is to know what processes are essential for normal functioning, and it is in this sense that immunological paralysis provides a useful tool for the study of the immune response and immunological memory.

The tolerance induced by antigen administration with agents which non-specifically block the immune response such as radiation[127] and anti-metabolites,[63] like the unresponsiveness induced by perinatal administration of antigen and protein-overloading paralysis, is specific and lasts long after the characteristic effects of the treatment have worn off. Something, therefore, remains in an altered state as a result of the previous treatment and it seems natural to direct our first inquiry to the nucleic acids.

It is known, for example, that there is an increase in RNA synthesis during the immune response, both during the primary and during the secondary.[173-175] We might wonder as to the necessity of the increased synthesis during the secondary response since

radiation [74,75] and radio-mimetic drugs[62-65] affect it much less than they do the primary, as we have noted earlier, and as we would expect if, indeed, the fact that the animal was exposed to the antigen was "coded" at the time of the primary. The increase at the time of the secondary response may be explained on the basis of cellular proliferation.

Cottier et al[175] interestingly point out that the increase in RNA synthesis in the mouse spleen during the secondary response reaches its peak on the second day after stimulation while the rate of DNA synthesis in mouse spleen after anamnestic stimulation reaches its peak four days later, on the sixth day. Presumably, then, the RNA responds first and the DNA increase follows, both probably reflecting cellular proliferation.

With these changes in RNA synthesis during the immune response in mind, we thought it might prove informative to investigate the changes in rate of RNA synthesis at various times after injection of a paralytic dose of antigen and also to investigate whether this change was any more profound if a second paralytic dose was administered to an already unresponsive animal since, unlike the immune response, tolerance does not lend itself to the measurement of antibody titers to demonstrate a secondary response. (If there is no antibody detectible after paralysis, the level cannot go any lower after a second paralyzing injection!) We might note in this connection that Cinader and Dubert[176] found that animals in which partial paralysis (i.e. low but detectible antibody levels)

had been induced had, after a second dose of antigen, even lower titers of antibody (presumably not due merely to precipitation by the second dose of antigen).

Dresser[140] had found that the minimum intraperitoneal dose of bovine gamma-globulin (BGG) in saline necessary for inducing complete paralysis in mice is between 50 and 200 micrograms. This is approximately 10^{15} molecules (compare with Smith and Bridges'[157] estimate of 10^{10} molecules for maintenance of tolerance). Deitrich and Weigle,[155] noted that five of five mice were rendered fully tolerant after intraperitoneal (IP) injections of 10, 1, 0.5, or 0.1 mg. of human gamma-globulin (HGG), smaller amounts resulting in immune responses. They noted that this paralysis was specific, as has been observed before.

Dresser found that giving mice 2mg. of BGG intraperitoneally would induce paralysis such that if BCG was administered subcutaneously with Freund's adjuvant, no immune response would occur to it. This effect (i.e. unresponsiveness) does not occur until three to five days after the administration of the paralyzing dose. Thus, many animals challenged three days after administration of the paralyzing dose will react with the formation of antibody, but at five days, four out of five were found to be unresponsive, and five of five at twelve days.

In our experiments, we examined the rate of RNA synthesis in the spleens of paralyzed animals as reflected by the uptake of H^3-cytidine, one of the four bases found in RNA. We em-

had been [illegible], a second case of [illegible] [illegible] of antibody [illegible] [illegible]

[illegible]

[illegible] of bovine [illegible] [illegible] [illegible] noted that five of [illegible] were reversed [illegible] [illegible] respectively [illegible]

[illegible]

[illegible]

ployed large doses of BGG in saline without adjuvant in addition to radiation in order to induce tolerance. The radiation was delivered by means of radioactive phosphorous (P^{32}) in the form of inorganic phosphate, as will be detailed under "Materials and Methods." In another experiment, we investigated paralysis induced by P^{32} irradiation, soluble antigen in large quantities, and radio-mimetic drugs. The addition of radio-mimetic drugs seemed to add little to the effects already produced by the irradiation and the large doses of antigen.

We would like to point out that while P^{32} has been used in the treatment of polycythemia vera because of its predilection for marrow and bones, it has never been used before in the induction of tolerance.

VIII. Materials and Methods

White female CFW strain mice weighing 19-23 grams (carworth Farms, New City, New York) were used for all experiments. Bovine gamma-globulin (BGG Armour and Company, Lot C-904) was dissolved in normal saline (Abbot Laboratories) to give a concentration of 4 mg. per milliliter. Solutions of 8-azaguanine (Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.) 0.6 mg. per milliliter, and 5-Bromouracil (5 BU, Cal Biochem) 6.5 mg. per milliliter in physiologic (0.89 Normal) saline were prepared.

The mice were divided initially into four groups of 21 animals, numbered one to four. On the first day of the experiment, all animals

received 60 microcuries of P^{32} given as inorganic phosphate (Oak Ridge National Laboratory, Oak Ridge, Tenn.) in 0.5 ml. of saline intraperitoneally. (This is about twenty times the therapeutic dose on a mC per kg. basis as is used in humans to treat polycythemia vera rubra.[177,178]) On this same day, one hour later, each animal in groups 2 - 4 received 2 mg. BGG in 0.5 ml. of saline, those in group 3 receiving in addition 0.5 ml. (0.3 mg.) of the 8-azaguanine solution, and those in group 4 receiving 0.5 ml. (3.25 mg.) of the 5-bromouracil solution. Groups 3 and 4 had received their respective anti-metabolites for two days preceding the administration of antigen and received the above dosage intraperitoneally every other day until the twentieth day after which these drugs were discontinued so that they would not interfere with incorporation of tritiated cytidine (H^3-cytidine).

Two more milligrams of BGG were administered to mice in groups 2 - 4 on the second day for a total of 4 milligrams of soluble BGG in saline, given intraperitoneally in a period of two days. As noted, all of these animals had also received a radiating dose of P^{32}.

The P^{32} was given primarily not as a tracer but to enhance the tolerance producing effects of a dose of antigen which in itself should be sufficient to produce paralysis for a finite period of time.[140] While it would have been possible to examine nucleic acid metabolism using P^{32} as a tracer, such examination was more carefully made by using H^3-cytidine which is a superior tracer for this purpose, both because it is more specific and because there are almost no half-

life errors involved, the half life of H^3 being 12.5 years, while that of P^{32} is 14.3 days.

Twenty-six days after the administration of P^{32} and BGG to groups 2 - 4 (and only P^{32} to group 1) all animals were given 0.25 ml. IP of a 2.5 mg./ml. solution of thephorin to prevent anaphylaxis* when antigen was subsequently administered. Three animals were then randomly selected from each group (1 - 4) to serve as controls for the balance of the experiment. The remainder of the animals (including those in group 1 which had received no antigen the first time) were given 2 mg. of BGG in 0.5 ml. of saline, also IP one-half hour following the thephorin.

The animals were sacrificed (two from each group) at 8½ hours, 1 day, 2 days, 3 days, 4 days, 7 days, 8 days, 9 days, and 10 days after the administration of antigen. The three animals which had been selected from each group to serve as controls received no antigen; it was therefore immaterial on which day they were sacrificed. To improve the quality of the control, however, four mice, i.e. one mouse from each of the four sub-groups, were sacrificed at the same time as those who were killed 1,7, and 9 days after the administration of antigen. As expected, however, since these animals received no antigen, the time of sacrifice did not affect any parameter which we studied.

Each mouse was given an intraperitoneal injection of 50 microcuries of H^3-cytidine (0.85 Curies per millimole, Schwarz Bioresearch

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* No clear-cut cases of anaphylaxis were seen, although 4 of the 72 experimental animals died shortly after injection of antigen, one of which was due to aortic trauma and hemoperitoneum; the cause of death in the others was undetermined.

like strong interactions, the half life [illegible]

than [illegible] 32 is [illegible].

Many-body [illegible] 32 [illegible]

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Inc., Mt. Vernon, N. Y.) in 0.5 ml. of normal saline four hours prior to sacrifice. The animals were killed with ether anesthesia, and as much blood as possible was removed by cardiac puncture. The spleens were removed and dried at 0° C in a vacuum line at 10^{-4} mm of mercury overnight. Spleens were used because it has been stated that although only 1% (approximately) of the antigen finds its way to the spleen, about 90% of the antibody has been shown in some studies[179] to come from that organ. The spleens were then weighed, homogenized with a Ten Broeck homogenizer in 0.3 N perchloric acid (0.125 ml. per mg. dry weight) and their RNA extracted according to the method of Schmidt and Thannhauser.[180]

A 1 ml. aliquot of the perchloric acid homogenate (equivalent to 8 mg. of dried tissue) was used for analysis. It was centrifuged at 0° C and then washed with 1 ml. of 0.3 M perchloric acid. It was then centrifuged again, washed again with perchloric acid, and then washed with 2 ml. of 96% ethanol followed by 2 ml. of a 3:1 96% ethanol-ether mixture. It was then washed with 2 ml. of a 1:1 methanol-ether mixture, and lastly, with 2 ml. of diethyl ether. After centrifuging and discarding the supernatant, the pellet was hydrolyzed in 1 ml. of 1 N potassium hydroxide at 25° C for 2½ hours after which the solution was neutralized with 0.2 ml. of 6 N perchloric acid. The resulting mixture was centrifuged, leaving the hydrolyzed RNA in the supernatant.

The RNA hydrolysate was then counted for H^3 by placing 0.1 ml. of the hydrolysate into a standard scintillation mixture for aqueous

Inc., Mt. Vernon, [illegible] normal saline [illegible] prior to sacrifice. The animals were [illegible] much blood as possible was [illegible] were removed [illegible] overnight. [illegible] were used because [illegible] only 1% (approximately) of the [illegible] about 70% of the [illegible] from that organ. [illegible] dry weight, and their RNA [illegible]

... 1 ml. aliquot of the [illegible] to 8 ms. of dried tissue [illegible] at 0° C and then [illegible]

washed with 1 ml. of 95% ethanol [illegible] ethanol-ether mixture. [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] phosphate RNA in the supernatant.

The RNA phosphate was then [illegible]

solutions consisting of 12 ml. of 0.3% PBD (Pilot Chemical Company) in xylene (Merck and Company) and 3 ml. of absolute ethanol to aid miscibility of the aqueous hydrolysate and the hydrophobic xylene. A Tri-carb scintillation counter was used. All samples were counted for twenty minutes or 10^6 counts. Efficiency, using a standard, was found to be 22.4% (which is rather high for an aqueous system).

Total RNA was then determined by taking another 0.1 ml., diluting to 3.1 ml. with triple distilled water, and reading optical density at 2600Å (260 mμ) in a Cary spectrophotometer. A Cary was used rather than a Beckman in order to observe the entire spectrum in this range so that we might be assured of the purity. An example of a typical plot may be seen in Fig. 3 in the Appendix.

From the raw parameters obtained viz. weight, H^3-cytidine incorporated per mg. of tissue, and RNA per mg., several other interesting parameters can be calculated. For example, total H^3-cytidine incorporated per spleen, total RNA (in moles) per spleen, and H^3-cytidine incorporated per mole of RNA. The calculations involved in obtaining the amount of RNA from the O.D. (optical density) readings are presented in the appendix.

The blood removed at sacrifice was centrifuged and the serum was subsequently used for antibody determinations.

IX. Critique of Experiments

Dresser[140] centrifuged his BGG at 20,000 to 30,000 g to remove particulate matter, noting that failure to do so might lead to sporadic immune responses. We did not centrifuge our BGG because

solutions containing [illegible] 12 ml. of 0.5% PPO/POPOP (Packard Chem. Co.), in xylene [illegible] 1 ml. of [illegible] miscibility of the aqueous homogenate and the [illegible]. A Tri-Carb scintillation counter was used. [illegible] counted for twenty minutes or 10⁴ counts. [illegible], was found to be 41.4% [illegible].

Total RNA was then determined by [illegible], diluting to 3.1 ml. with [illegible], and [illegible] optical density at 260 mμ in a Cary spectrophotometer [illegible] was used [illegible] the term in the range [illegible]. An example of a typical [illegible] Fig. 3 [illegible].

From the RNA parameters [illegible] incorporated per mg. of [illegible]. However, other interesting parameters can be calculated. [illegible] cytidine incorporated per organ, [illegible] and H³-cytidine incorporated per [illegible] involved in obtaining the [illegible] (optical density) readings are presented in [illegible].

The blood [illegible] was [illegible] serum was [illegible].

4. [illegible]

[illegible]

at the time of performing the experiments, Dresser's paper had not yet been published. In order to be maximally efficient in the production of tolerance, however, the BGG should be centrifuged as noted above, despite the fact that we and others [157,155,127] have obtained satisfactory results without doing so.

In addition, a second control group, one which had received neither antigen nor irradiation would add to the completeness of our data, although admittedly, what we are most interested in is the difference between animals paralyzed by antigen and animals not so paralyzed. That radiation alone is not responsible for the changes observed is confirmed by the fact that our control animals were also irradiated (see Fig. 2) and also supported by the fact that the changes were observed thirty-six days after the administration of the P^{32}, the biological half-life of which is only ten to eleven days. (Biological half-life is a measure of both radioactive decay and excretion.)

X. Results

It was found that the incorporation of H^3-cytidine into spleen RNA, and hence the rate of RNA synthesis after a paralyzing dose of antigen is strongly dependent upon the time elapsed after the administration of the soluble protein. It was also noted that the effects on RNA synthesis of a paralyzing dose of BGG were the same whether or not the animal had received a similar dose previously, and whether or not 5-bromouracil or 8-azaguanine had been given with and after the antigen. Our doses of both were, however, somewhat low and since the half-life in the body of the former is rather short, the two day

interval between doses may have resulted in ineffective blood levels. As noted before, the anti-metabolites had been discontinued one week prior to administration of the BGG.

In brief, the results obtained in groups 1, 2, 3, and 4 were all similar, in contrast to those of the control animals which received no antigen. Table 1 summarizes the changes in H^3-cytidine incorporation into spleen RNA which occur at various times after administration of 2 mg. of BGG to mice which had received 60 microcuries of P^{32} twenty-seven days earlier. The data from groups 1 - 4 have been averaged and compared with those from controls. The data from the individual animals may be found in the Appendix, Table 6.

As noted, Table 1 presents the dpm (disintegrations per minute) of H^3-cytidine per dry weight of spleen, but since the spleen weights varied, Table 2 presents the mean values for the total dpm of H^3-cytidine per spleen of experimental animals vs controls. Again, the data from individual animals can be found in the Appendix, Table 7. All data are given in the form of disintegrations per minute (dpm) rather than counts per minute (cpm) as they have been corrected for background and counting efficiency.

It is evident from both Table 1 and Table 2 that the H^3-cytidine incorporation into RNA and hence the rate of RNA synthesis is almost unaffected by a paralyzing dose of antigen until eight days have elapsed after the administration of antigen, at which time RNA synthesis almost ceases. (See Fig. 2)

Due to the large variations between animals we cannot say if the original increase of incorporation of H^3-cytidine peaking around the second day, which we see in these tables, is real or merely an

TABLE I

H^3-cytidine Incorporation per mg. Dry Weight of Spleen Averages of Experimental Animals (All Groups) vs Controls

	Time After 2 mg. BGG IP								
	8.5 hours	1 day	2 days	3 days	4 days	7 days	8 days	9 days	10 days
Mean dpm/mg. dry weight in exptl. animals ± S.E.*	13,972 1.59	22,497 2.37	28,882 4.03	17,816 2.32	14,621 2.14	11,578 1.39	2,115 0.24	282 0.05	416 0.05
No. of animals	7	8	7	8	8	8	8	6	7
Mean dpm/mg. dry weight in control animals ± S.E.*		19,468 2.39				21,380 29.45**		15,795 4.58	
No. of animals		3				4		3	

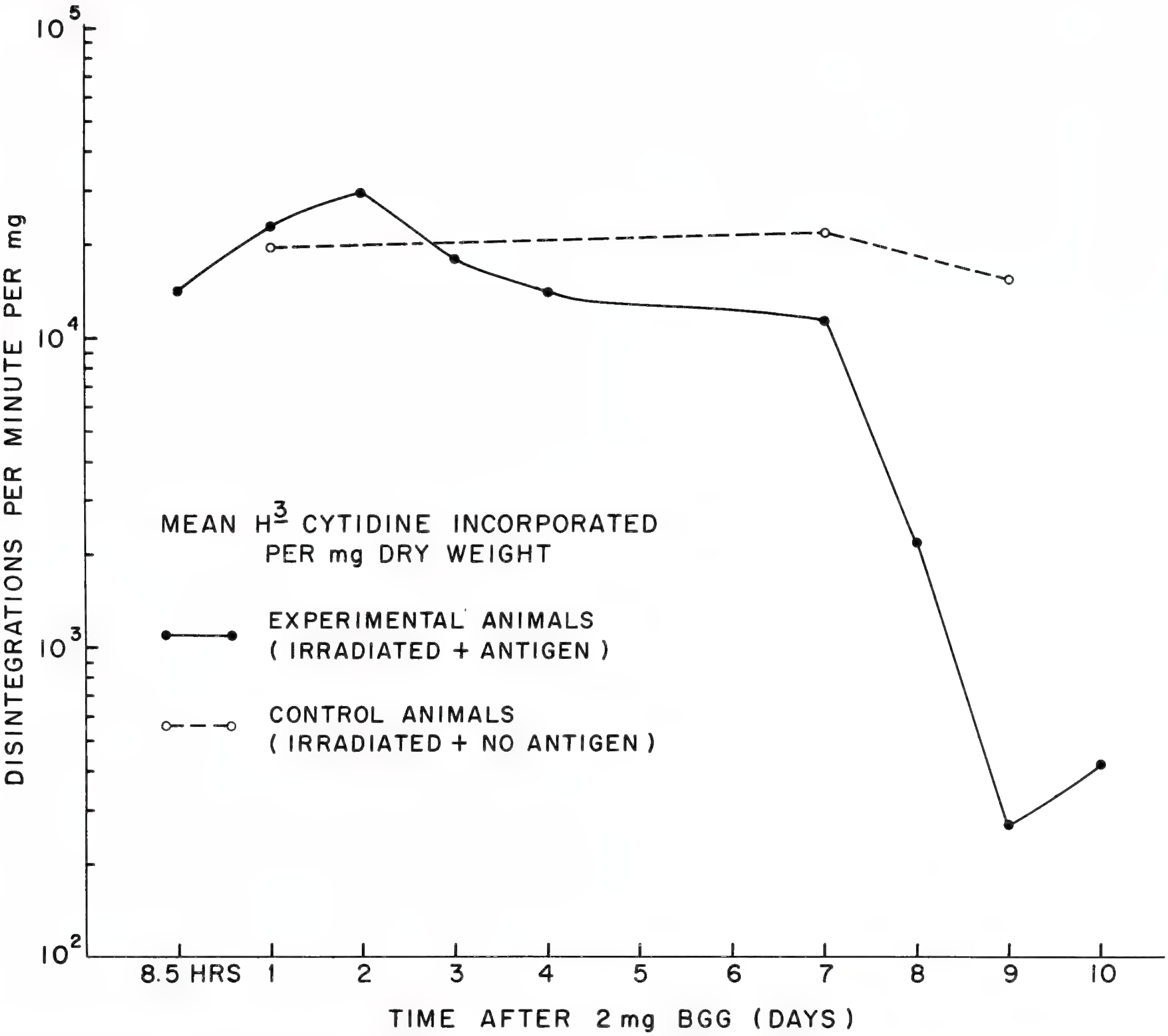
* Standard error of the mean is given in thousands; thus, for example, 1.59 signifies 1,590.

** This is the result of one extremely high and one extremely low value.

TABLE 2

Total H^3-cytidine Incorporation per Spleen Averages of Experimental Animals (All Groups) vs Controls

	Time after 2 mg. BGG								
	8.5 hours	1 day	2 days	3 days	4 days	7 days	8 days	9 days	10 days
Mean total dpm/whole spleen in exptl. animals ± S.E.*	449,711 89.0	533,364 62.0	753,851 170.3	841,863 211.6	412,607 64.8	340,970 33.3	51,941 3.7	10,624 1.7	11,990 1.6
No. of animals	7	8	7	8	8	8	8	6	7
Mean total dpm/whole spleen in control animals ± S.E.*		628,769 70.2				289,290 129.5		445,675 250.4	
No. of animals		3				4		3	



10^5
10^4
10^3
10^2
DISINTEGRATIONS PER MINUTE PER mg
MEAN H^3 CYTIDINE INCORPORATED PER mg DRY WEIGHT
EXPERIMENTAL ANIMALS (IRRADIATED + ANTIGEN)
CONTROL ANIMALS (IRRADIATED + NO ANTIGEN)
8.5 HRS
1
2
3
4
5
6
7
8
9
10
TIME AFTER 2 mg BGG (DAYS)

FIGURE 2

artifact. Similarly, we are wary of offering an interpretation of the slight decrease in tritiated cytidine incorporation between the second and seventh days, as this too may or may not be an artifact.

The marked decrease in H^3-cytidine incorporation between the seventh and eighth days, however, and the even more marked drop between the eighth and ninth day greatly exceed the limits of biological variation and signify a great decrease in the rate of synthesis of RNA. There is what appears to be a change in the direction of recovery on the tenth day, but again, one cannot be sure. Unfortunately, the animals were not followed beyond ten days after antigen administration.

It is interesting to note that despite the marked decrease in RNA synthesis, the measured RNA per mg. and RNA per spleen remain almost unchanged, as determined by $O.D._{260m\mu}$. (Tables 3 and 4)

Since the RNA per mg. remains relatively constant in all animals while the H^3-cytidine incorporation per mg. decreases steadily in animals sacrificed from the first to the ninth day, the H^3-cytidine incorporation per mole of RNA (Table 5) follows the same general pattern as the H^3-cytidine incorporation per mg. (Tables 1 - 5 are summary tables, and detailed tables which include data for each individual animal will be found in the Appendix, i.e. Tables 6 - 11)

The sera collected at sacrifice were analyzed for antibody by complement fixation and all were negative for antibody at 1:20 dilutions.

XI. Discussion and Conclusions

The changes in the rate of synthesis of splenic RNA are

artifact. Similarly, we are wary of attaching much importance to the [illegible] slight decrease in [illegible] output [illegible] and seventh day, as this too may or may not be [illegible].

The marked decrease in ^{3}H-[illegible] seventh and eighth days, however, [illegible] between the eighth and ninth day, [illegible] typical variation and signify a [illegible] it was. There is what appears to be [illegible] recovery on the ninth day, [illegible] the animals were not followed beyond the [illegible]

It is interesting to note that [illegible] and synchrony, [illegible] almost [illegible]

Since the [illegible] while the ^{3}H-thymidine incorporation [illegible] animals [illegible] from the first [illegible] incorporation per mole [illegible] pattern as the ^{3}H-thymidine [illegible] are [illegible] tables, [illegible] individual animal will [illegible]

As data collected [illegible] [illegible] function and adjacent [illegible]

[illegible]

The changes in the [illegible]

T A B L E 3

Mean Values for RNA in Moles x 10^{7*} per mg. Dry Weight**
Averages of Experimental Animals (All Groups) vs Controls

Time after 2 mg. BGG IP

	8.5 hours	1 day	2 days	3 days	4 days	7 days	8 days	9 days	10 days
Experimental animals	1.40	1.38	1.64	1.36	1.43	1.31	1.28	1.27	1.22
No. of animals	7	8	7	8	8	8	8	6	7
Control animals		1.24				1.41		1.26	
No. of animals		3				3		3	

* i.e. values are to be multiplied by 10^{-7} to obtain moles of RNA/mg. dry weight
** calculations for this table are discussed in the Appendix with the data from individual animals

T A B L E 4

Mean Values for Total RNA per Spleen in Moles x 10^{7*}
Averages of Experimental Animals (All Groups) vs Controls

Time after 2 mg.BGG IP

	8.5 hours	1 day	2 days	3 days	4 days	7 days	8 days	9 days	10 days
Experimental animals	44.24	32.59	42.66	71.98**	37.56	40.73	34.54	51.55	35.37
No. of animals	7	8	7	8	8	8	8	6	7
Control animals		39.92				40.16		30.51	
No. of animals		3				3		3	

* i.e. values are to be multiplied by 10^{-7} to obtain moles of RNA/spleen
** this is due to 2 extremely large spleens, without which the average would be 35.17

TABLE 5

Mean Values for H^3-cytidine Incorporation per 10^{-7} Moles of Spleen RNA

Averages of Experimental Animals (All Groups) vs Controls

	8.5 hours	1 day	2 days	3 days	4 days	7 days	8 days	9 days	10 days
Experimental animals	9,840	15,909	18,676	11,235	10,684	8,938	1,604	262	335
No. of animals	7	8	7	8	8	8	8	6	7
Control animals		15,975				9,681		12,398	
No. of animals		3				4		3	

interesting with regard both to their magnitude and to the latent period between administration of antigen and the time at which they are observed. As might be expected, despite the great alterations in rate of RNA synthesis, the total amount of RNA per mg. varies little, since our method of analysis treats all RNA together including ribosomal RNA which, compared to mRNA, is present in considerably larger quantity and is much more stable.

As noted earlier, Dresser[140] showed that in his animals unresponsiveness was only truly established between three and five days after antigen administration. If the unresponsiveness in the case of Dresser's animals was due to decreased RNA synthesis, as we found, one must ask how can the result become manifest before the cause, viz. the response is evident at three to five days, and the decreased synthesis is seen only at eight to nine days. To this question we do not have an answer except for the possible difference between strains, which seems unlikely, but if we were to speculate from our results and those of Liacopolos et al,[143] perhaps there are two types of paralysis - a specific type and a non-specific type. The specific type would not require a generalized decrease in RNA synthesis, while such a large decrease in RNA synthesis could explain generalized, non-specific unresponsiveness. We might note, too, that Liacopolos et al found the non-specific unresponsiveness to commence approximately ten days after the first paralyzing antigen dose, roughly the same time after antigen administration that we noticed the decrease in RNA synthesis. Let us recall, however, that his animals were guinea pigs and ours were mice.

We pointed out earlier that if sufficient DNA were incapacitated by complexing with antigen, this could possibly account for decreased RNA synthesis, but we are still at a loss to even postulate an explanation for the long lag period of about ten days. Perhaps the reaction of antigen complexing with DNA, if it occurs at all, is a very slow one, a small amount of intracellular antigen first complexing with that DNA which has the greatest affinity for it (thereby causing specific tolerance) and more antigen subsequently complexing with other DNA (thereby resulting in non-specificity). That the earliest establishment of any paralysis takes three to five days, as shown by Dresser[140] would support our postulation of the slowness of whatever reaction occurs. Since the paralysis again becomes specific, as claimed by Liacopolos et al,[143] perhaps a decrease of available antigen due to catabolism, etc. results in the break up first of the complexes which have the least stability, i.e. in the reverse order of their formation. We must re-emphasize, however, that these are only speculations - admittedly, speculations which fit the presently available data well - but we must realize that we might be building a house of cards.

In a preliminary confirmatory study, we repeated our experiments, administering 2 mg. of soluble BGG IP to the same strains of mice, but without prior P^{32} irradiation. (Table 11 in the Appendix) These animals did not show the changes in H^3-cytidine incorporation which were so manifest in our earlier study. Unfortunately, we did not do antibody titer determinations on these animals, and since it is known that radiation prior to the antigenic stimulation greatly enhances unresponsiveness, perhaps these animals

were not rendered completely tolerant. Further studies in irradiated animals are therefore indicated.

Since our data on the non-irradiated animals is less complete, (fewer parameters were studied, fewer animals were used, and the conditions were less auspicious for the production of tolerance), we shall confine our remarks for the present to the larger group with which we have been dealing.

Our work, showing the decrease in the rate of RNA synthesis in the spleens of immunologically paralyzed animals (mice) serves almost as a companion experiment to the work of Feldman et al[146] which showed that nucleic acid administration can end a period of tolerance. To repeat our earlier speculation, we believe that tolerance is not a positive phenomenon, but a competitive blockage of nucleic acid synthesis by the antigen obstructing access to the DNA, within cells. This block can be overcome, however, as can any competitive inhibition by excess reagent, i.e. by adding nucleotides and small polymers of nucleotides, (much as the inhibition of prothrombin and factor VII synthesis by dicumarol can be overcome by large doses of vitamin K). As proposed, this may act by displacing the antigen from the surface of the DNA.

This would imply, then, that tolerance is due to a metabolic disturbance and not due to cellular elimination (as proposed by Burnet and Lederberg[92,99,100-102,104,117]), or to increased production of "immunologically incompetent lymphocytes" as proposed by Gorman and Chandler.[172]

The profound changes in the rate of RNA synthesis would, in sum, certainly support the hypothesis that RNA is one of the substances involved when the body becomes unresponsive to an antigen, even if its role is, as postulated, only passive. Whether RNA is

the final engram in which this information is coded is still undetermined, but since we noted no increase, but rather a decrease in RNA synthesis, it seems unlikely. It is, however, still possible that some old RNA might become rearranged without any net synthesis to code the unresponsiveness, in spite of our findings. Nevertheless, this also seems unlikely since if old RNA were to dissociate, we would expect our labelled precurser to mix with the pool of cellular cytidine, some of which should therefore be incorporated as a reflection of any increase in RNA metabolism, even without net synthesis.

In summary, one more tiny piece has been added to the great puzzle of immunological tolerance, but the final picture still awaits many more and larger pieces.

APPENDIX

FIGURE 3

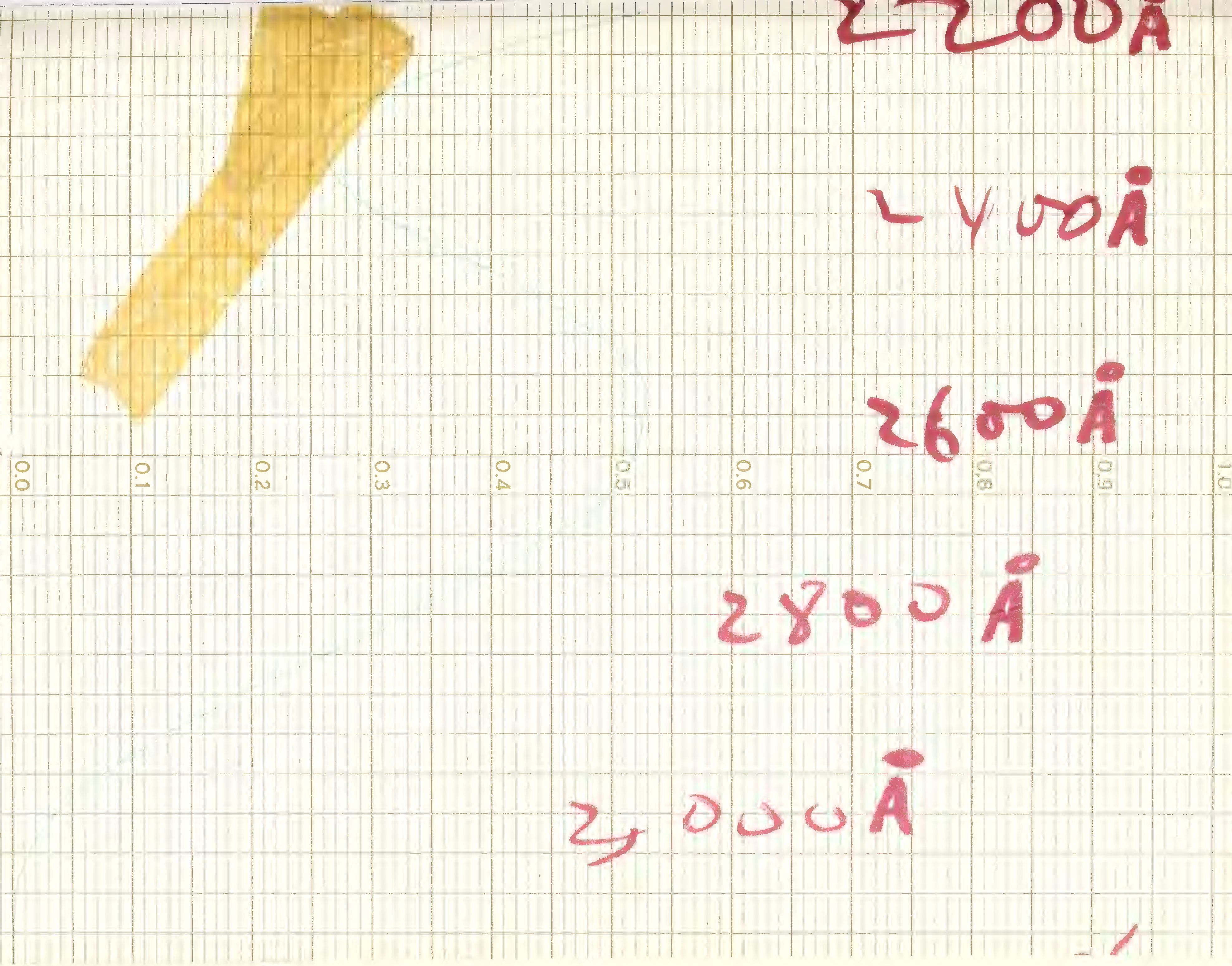


TABLE 6

H^3-cytidine Incorporation in dpm per mg. Dry Weight of Spleen

Time after antigen	Animal							
	I_1	I_2	II_1	II_2	III_1	III_2	IV_1	IV_2
8.5 hours	15,162	6,718	16,364		9,995	18,252	17,343	13,968
1 day	23,309	17,894	13,975	30,575	21,302	30,563	14,665	27,695
2 days		40,283	12,941	29,806	29,665	42,944	19,011	27,526
3 days	20,071	27,856	12,153	17,655	9,768	25,418	11,357	18,254
4 days	17,025	21,896	17,545	16,159	15,521	1,036	13,890	13,897
7 days	9,958	9,360	18,066	6,803	10,314	17,000	11,755	9,375
8 days	1,712	1,797	1,963	1,451	1,661	1,907	3,670	2,160
9 days	321	379	500	317	325		130	
10 days	183	450	436	587	366	510	380	
CONTROLS:	I		II		III		IV	
1 day	14,766		22,532				21,105	
7 days	15,084		45,530		23,820		1,089	
9 days	13,695				24,565		9,125	

TABLE 7

Total H^3-cytidine Incorporated in dpm per Whole Spleen

Time after antigen	Animal							
	I_1	I_2	II_1	II_2	III_1	III_2	IV_1	IV_2
8.5 hours	328,257	165,934	837,018		217,891	449,911	607,005	541,948
1 day	600,207	391,878	309,057	834,697	471,839	696,836	397,421	564,978
2 days		613,913	332,583	733,227	528,037	1,730,643	643,522	695,031
3 days	556,970	956,854	1,274,728	304,372	290,109	2,029,627	382,162	940,081
4 days	482,658	626,444	502,664	523,228	435,364	20,564	397,948	311,988
7 days	286,989	275,652	394,019	191,368	299,622	446,930	365,463	467,719
8 days	57,180	45,553	42,401	39,685	47,255	51,107	70,464	61,884
9 days	14,525	9,770	14,075	16,516	13,159		6,323	
10 days	4,538	12,870	11,423	13,049	17,941	15,121	8,987	

CONTROLS:	I	II	III	IV
1 day	504,111	746,936		635,260
7 days	362,016	135,984	620,511	38,649
9 days	295,812		934,453	106,762

TABLE 8

RNA per mg. Dry Weight in Moles x 10^{7*}

Time after antigen	Animal I_1	I_2	II_1	II_2	III_1	III_2	IV_1	IV_2
8.5 hours	1.2816	1.2042	1.4536		1.2945	1.4665	1.4794	1.5912
1 day	1.3160	1.5396	1.4149	1.4966	1.5525	1.1999	1.2859	1.6256
2 days		1.7246	1.4580	1.7547	1.5998	1.1440	2.1373	1.6644
3 days	1.0838	2.2363	1.8923	1.2386	1.1182	1.5224	1.5697	
4 days	1.4278	1.4923	1.3891	1.2386	1.4665	1.5181	1.4063	1.1526
7 days	1.2902	1.4149	1.2828	1.0279	1.2257	1.3246	1.4020	1.5568
8 days	1.3031	1.2816	1.6256	1.2214	1.0752	1.1569	1.3461	1.1999
9 days	1.3246	1.2214	1.1483	1.4665	1.2601		1.1956	
10 days	0.8042	1.3246	1.2859	1.4966	1.1956	1.2128	1.1956	

CONTROLS:	I	II	III	IV
1 day	1.2343	1.0924		1.3762
7 days	1.4192		1.4536	1.3676
9 days	1.3332		1.2987	1.1397

* these figures are to be multiplied by 10^{-7} to obtain moles RNA/mg. dry weight

Method for Obtaining RNA values from $OD_{260m\mu}$

$OD = \log I/I_o = c\lambda\varepsilon$

where c = the concentration of solute in moles per liter
λ = the length of the light path in centimeters
ε = the molar extinction coefficient

$C_{in\ M/L} = OD/\varepsilon$

In our case, $\lambda = 1$.

$C_{in\ M/ml.} = 10^{-3}\ OD/\varepsilon$

Continued on next page.

Since we diluted 0.1 ml. to 3.1 ml., the actual concentration, $C_{actual} = 31\ C_{measured}$

Hence,

$$C_{actual} = 10^{-3} \times 31 \times OD/\varepsilon$$

This 0.1 ml came from a total of 1.2 ml. of solution which, it will be recalled represented 8 mg. of dry tissue. Thus, 0.15 ml. would represent 1 mg. dry weight. We must therefore multiply our concentration in moles per ml. by 0.15 to obtain moles per mg.

Therefore,

$$\text{Moles per mg} = 3.1 \times 10^{-2} \times 0.15 \times OD/\varepsilon$$

Depending on the source,[181,182] the values of ε_{260}^{RNA} at pH 7 (which we neutralized our samples to) are given as between 10,000 and 10,816. We assumed the latter value. Thus, our formula becomes:

$$\text{RNA in Moles/mg. dry weight} = 4.30 \times 10^{-7}\ OD_{260m\mu}$$

TABLE 9

Total RNA per Spleen in Moles x 10^7*

Time after antigen	Animal I_1	I_2	II_1	II_2	III_1	III_2	IV_1	IV_2
8.5 hours	27.74	29.74	74.35		28.22	36.14	51.77	61.73
1 day	33.89	33.72	31.29	40.86	25.59	27.36	34.85	33.16
2 days		26.28	37.47	43.17	28.48	46.10	72.35	42.03
3 days	30.08	38.41	198.48	21.35	33.21	129.55	52.82	
4 days	40.48	42.69	39.80	40.11	41.14	30.13	40.29	25.88
7 days	37.18	41.67	26.45	28.91	43.59	77.67	35.61	34.82
8 days	43.52	32.49	35.11	33.41	30.59	31.00	25.85	34.38
9 days	59.94	31.49	32.32	76.40	51.02		58.15	
10 days	19.94	37.88	33.69	33.27	58.61	35.96	28.28	

CONTROLS:	I	II	III	IV
1 day	42.14	36.21		41.42
7 days	34.06		37.87	48.54
9 days	28.80		49.40	13.33

* i.e. these values must be multiplied by 10^{-7} to obtain moles of RNA/whole spleen

TABLE 10

H^3-cytidine Incorporation in dpm per 10^{-7} Moles of RNA

Time after antigen	Animal							
	I_1	I_2	II_1	II_2	III_1	III_2	IV_1	IV_2
8.5 hours	11,830	5,578	11,257		7,721	12,445	11,723	8,778
1 day	17,712	11,622	9,877	20,430	13,721	25,471	11,404	17,037
2 days		23,358	8,876	16,986	18,543	37,538	8,894	16,538
3 days	18,519	12,456	6,422	14,253	8,735	16,696	7,235	5,563
4 days	11,924	14,673	12,630	13,046	10,584	682	9,877	12,057
7 days	7,718	6,615	14,896	6,618	8,415	12,834	8,384	6,022
8 days	1,314	1,402	1,208	1,188	1,545	1,648	2,726	1,800
9 days	242	310	435	216	258		109	
10 days	227	340	339	392	306	421	318	

CONTROLS:	I	II	III	IV
1 day	11,963	20,626		15,336
7 days	10,629	10,914	16,387	796
9 days	10,272		18,915	8,006

TABLE 11

H^3 Cytidine Incorporation into RNA and DNA after 2 mg. BGG IP without prior P^{32}

	Days after Antigen							
Animal	1 Day	6 Days	8 Days	9 Days	10 Days	11 Days	15 Days	Control No Antigen
#1								
RNA H^3 dpm/mg dry wt.	8,990	14,255	5,439	637	8,606	12,930	9,238	10,014
Total RNA H^3 dpm/whole spleen	228,796	308,427	138,966	14,854	231,501	530,480	381,066	327,958
DNA H^3 dpm/mg dry wt.	954	1,042	435	283	710	2,707	1,618	850
Total DNA H^3 dpm/whole spleen	24,279	29,124	11,114	6,600	19,099	107,062	66,742	27,832
RNA H^3/DNA H^3	9.42	13.68	12.50	2.25	12.12	4.78	5.71	11.78
#2								
RNA H^3 dpm/mg dry wt.	4,575	10,294	8,529		5,597	5,396	8,028	10,710
Total RNA H^3 dpm/whole spleen	110,715	253,232	231,562		192,817	229,953	230,403	357,704
DNA H^3 dpm/mg dry wt.	402	3,042	461		821	910	648	806
Total DNA H^3 dpm/whole spleen	9,728	74,833	12,516		28,283	38,993	18,598	26,920
RNA H^3/DNA H^3	11.38	3.38	18.50		6.82	5.90	12.39	13.28
#3								
RNA H^3 dpm/mg dry wt.	6,907	7,450	4,268	13,610	5,120	7,537	6,388	6,888
Total RNA H^3 dpm/whole spleen	139,180	183,270	108,834	270,158	196,608	241,938	312,054	250,379
DNA H^3 dpm/mg dry wt.	656	1,213	412	1,601	478	767	559	856
Total DNA H^3 dpm/whole spleen	13,210	29,840	10,506	31,779	18,355	24,621	27,307	31,116
RNA H^3/DNA H^3	10.52	6.14	10.36	8.50	10.71	9.82	11.42	8.05

Continued on next page

Table 11 Continued

nimal	1 Day	6 Days	8 Days	9 Days	10 Days	11 Days	15 Days	Control No Antigen
4								
NA H^3 pm/mg dry wt.	14,299		8,784	5,067	11,078	11,746		8,559
otal RNA H^3 pm/whole spleen	318,868		262,202	119,125	350,065	589,062		402,701
NA H^3 pm/mg dry wt.	852		559	603	792	373		692
otal DNA H^3 pm/whole spleen	19,000		16,686	14,177	25,027	18,706		32,559
NA H^3/DNA H^3	16.78		15.71	8.40	13.99	31.49		12.37
5								
NA H^3 m/mg dry wt.								10,573
tal RNA H^3 m/whole spleen								267,497
A H^3 m/mg dry wt.								892
tal DNA H^3 m/whole spleen								22,567
A H^3/DNA H^3								11.85
an RNA H^3 l animals m/mg.	8,693	10,666	6,755	6,438	7,600	10,057	7,885	9,349
an DNA H^3 l Animals m/mg.	716	1,766	467	829	698	1,189	941	819

BIBLIOGRAPHY

1. Silverstein, A. M. (1964): Immunologic and Psychic Memory, *Neurosciences Research Program Bull. 1*, No. 1, p.1.
2. Matthaei, J. H., Jones, D. W., Marttin, R. G., and Nirenberg, M. W. (1962): Character and Compostion of RNA Coding Units, *Proc. Nat. Acad. Sci. 48*, 666.
3. Jones, D. W., and Nirenberg, M. W. (1962): Qualitative Survey of RNA Codewords, *Proc. Nat. Acad. Sci. 48*, 2115.
4. Humphrey, G. and Coxen, R. V. (1963): *The Chemistry of Thinking*, Chas. C. Thomas, Springfield, Ill.
5. Benacerraf, B., Ojeda, A., and Maurer, P. (1963): Studies on Artificial Antigens: II. The Antigenicity in Guinea Pigs of Arsanilic Acid Conjugates of Co-polymers of D- and L-alpha amino acids, *J. Exp. Med. 118*, 945.
6. Arquilla, E. and Fian, J. (1963): Genetic Differences in Antibody Production to Determinant Groups on Insulin, *Science 142*, 400.
7. Silverstein, A. M. (1964): Ontogeny of the Immune Response, *Science 144*, 1423.
8. Uhr, J. (1963): Actinomycin D: Its Effect on Antibody Formation in Vitro, *Science 142*, 1476.
9. Hydén, H. (1964): Introductory Remarks to the Session on Memory Processes, *Neurosciences Research Program Bull. II*, No. 3,23.
10. Lawrence, H. S. (1959): The Transfer of Hypersensitivity of the Delayed Type in Man, in *Cellular and Humoral Aspects of Hypersensitivity States*, H. S. Lawrence, ed., Hoeber - Harper, New York.
11. Uhr, J. (1964): Heterogeneity of the Immune Response, *Science 145*, 457.
12. Benedict, A. C., Larson, C., and Nik-khah, H. (1963): Synthesis of Antibody of High and Low Molecular Weight, *Science 139*, 1302.
13. Uhr, J. W., and Finkelstein, M. S. (1963): Antibody Formation: IV. Formation of Rapidly and Slowly Sedimenting Antibodies and Immunological Memory to Bacteriophage Ø X174, *J. Exp. Med. 117*, 457.
14. Bauer, D. C., Mathies, M. J. and Stavitsky, A. B. (1963): Sequences of Synthesis of Gamma-1 Macroglobulin and Gamma-2 Globulin Antibody During Primary and Secondary Responses to Proteins, Salmonella Antigens and Phage, *J. Exp. Med. 117*, 889.

15. Hechter, O. and Halkerston, I. K. (1964): On the Nature of Macromolecular Coding in Neuronal Memory, *Perspectives in Biol. and Med. 7*, 183.

16. Luk'ianenko, V. I. (1962): Result of Physiological Analysis of the Process of Immunogenesis with Reference to its Conditioned Reflex Regulation, *Izv. Akad. Nauk SSR (Biol) 4*, 592.

17. Luk'ianenko, V. I. (1961): The Functional Structure of the Immunogenetic Process and its Nervous Regulation, *Folia Biol. (Praha) 7*, 379.

18. Krylov, V. N., and Malinovskii, O. V. (1961): Dynamics of the Formation of Agglutinins in Relation to the Functional Force of Neural Processes, *Zh. Mikrobiol. 32*, 92.

19. Korneoa, E. A., and Khai, L. M. (1961): On the Role of the Sympatho-adrenal System in the Regulation of Immunogenic Processes, *Fiziol. Zh. SSR Sechenov 47*, 1298.

20. Petrovskii, I. N. (1961): Problems of the Nervous Regulation of Immunity Reactions: I. The Influence of Stimulation of Various Regions of the Brain on Agglutinin Titer, *Zh. Mikrobiol.* (in English) *32*, 1304.

21. Petrovskii, I. N. (1962): Influence of Experimental Neuroses on Immunity Reactions, *Zh. Mikrobiol. 32*, 1451.

22. Il'inskii, Y. A. (1962): Immunologic Reactions in Schizophrenic Patients Treated with Reserpine, *Vestnik Akad. Med. Nauk SSR 17*, 43.

23. Roberts, R.B. (1964): Self Induction and Memory, *Neurosciences Research Program Bull. II*, No. 3, 39.

24. Lorrente de Nó, R. (1962): Circulation of Impulses and Memory, in *Macromolecular Specificity and Biological Memory*, F. O. Schmitt, ed., MIT Press, Cambridge, Mass.

25. Schmitt, F. O. (1964): Molecules and Memory, *New Scientist 23*, 643.

26. Morowitz, H. J. (1964): Memory Storage and Low Temperature Biology, *Neurosciences Research Program Bull. II*, No. 4, 25.

27. Dingman, W. and Sporn, M. (1964): Molecular Theories of Memory, *Science 144*, 26.

28. Gerard, R. W., Chamberlain, T. J., and Rothschild, G. G. (1963): RNA in Learning and Memory, *Science 140*, 381.

29. Adey, W. R. (1963): Physicochemical Changes in Brain Tissue in Learning; Possible Relationships of RNA to Memory, *Neurology 13*, 359.

30. Gaito, J. (1963): DNA and RNA as Memory Molecules, Psychol. Rev. 70, 471.

30a. Gaito, J. (1961): A Biochemical Approach to Learning and Memory, Psychol. Rev. 68, 288.

30b. Gaito, J. and Zavala, A. (1964): Neurochemistry and Learning, Psychol. Bull. 61, 45.

31. Schmitt, F. O. ed., Macromolecular Specificity and Biological Memory, MIT Press, Cambridge, (1962).

32. Fields, W. S. and Abbott, W. eds. (1963): Information Storage and Neural Control, Chas. C. Thomas, Springfield, Ill.

33. Weiner, N. (1964): Dynamical Systems in Physics and Biology, New Scientist No. 375, 211.

34. Hydén, H. and Egyházi, E. (1962): Nuclear RNA Changes of Nerve Cells in Learning Experiments in Rats, Proc. Nat. Acad. Sci. 48, 1366.

35. Hydén, H. and Egyházi, E. (1963):Glial RNA Changes During a Learning Experiment in Rats, Proc. Nat. Acad. Sci. 49, 618.

36. Hydén, H. (1962): The Neuron and its Glia; a Biochemical and Functional Unit, Endeavor 21, 144.

37. Hydén, H. (1961): Satellite Cells in the Nervous System, Sci. Amer. 205, No. 5, 62.

38. Morrell, F. (1962): Electrochemical Mechanisms and Information Storage in Nerve Cells, in Macromolecular Specificity and Biological Memory, F.O. Schmitt, ed., MIT Press, Cambridge.

39. Morrell, F. (1961): Electrophysiological Contributions to the Neural Basis of Learning, Physiol. Rev. 41, 443.

40. Corning, W.C., and John, E. R. (1961): Effect of Ribonuclease on Retention of Conditioned Response in Regenerated Planarians, Science 134, 1363.

41. Mc Connell, J. V. (1962): Memory Transfer Through Cannibalism in Planarians, J. Neuropsychiat. 3, Suppl. 1 S42.

42. Zelman, A., Kabot, L., Jacobsen, R. and Mc Connell, J. V. (1963): Transfer of Training through Injection of Conditioned RNA into Untrained Planarians, Worm Runner's Digest 5, 14.

43. Egyházi, E., Hydén, H., and John, E. R.: Unpublished data, cited in Hyden, H. (1964): Introductory Remarks to the Session on Memory Processes, Neurosciences Res. Prog. Bull. II, No. 3, 23 (ref. 9, above).

44. Geiger, A. (1957): Chemical Changes Accompanying Activity in the Brain, in *Metabolism of the Nervous System*, D. Richter, ed. Pergamon Press, London.

45. Cameron, D. E. (1963): The Processes of Remembering, *Brit. J. Psychiat.* *109*, 325.

46. Cameron, D. E. and Solyom, L. (1961): Effects of Ribonucleic Acid on Memory, *Geriatrics* *16*, 74.

47. Cameron, D.E., Solyom, L., and Beach, L. (1961): Further Studies upon the Effects of the Administration of Ribonucleic Acid in Aged Patients Suffering from Memory (Retention) Failure, *Neuropharm.* *2*, 351.

48. Cameron, D. E. (1958): The Use of Nucleic Acid in Aged Patients with Memory Impairment, *Am. J. Psychiatry*, *114*, 943.

49. Cook, L., Davidson, A. B., Davis, D. J., Green, H., and Fellows, E. J. (1963): Ribonucleic Acid: Effect on Conditioned Behavior in Rats, *Science* *141*, 268.

50. Dingman, W. and Sporn, M. B. (1961): The Incorporation of 8-azaguanine into Rat Brain RNA and its Effect on Maze Learning by the Rat: An Inquiry into the Biochemical Basis of Memory, *J. Psychiat. Res.* *1*, 1.

51. Chamberlain, T. J., Rothschild, G. H. and Gerard, R. W. (1963): Drugs Affecting RNA and Learning, *Proc. Nat. Acad. Sci.* *49*, 918.

52. Fishman, M. (1961): Antibody Formation in Vitro, *J. Exp. Med.* *114*, 837.

53. Fishman, M. and Adler, F. I. (1963): Antibody Formation Initiated in Vitro, *J. Exp. Med.* *117*, 595.

54. Fishman, M., Hammerstrom, R. A. and Bond, V. P. (1963): In Vitro Transfer of Macrophage RNA to Lymph Node Cells, *Nature* *198*, 549.

55. Mannick, J. A. and Egdahl, R.H. (1962): Ribonucleic Acid in Transformation of Lymphoid Cells, *Science* *137*, 976.

56. Šterzl, J. and Hrubesová, M. (1956): The Transfer of Antibody Formation by Means of Nucleoprotein Fractions to Non-immunized Recipients, *Folia Biol.* (*Praha*) *2*, 21.

57. Noro, Y. (1962): Antibody Formation Promoting Function of Lymphocyte RNA, *Jap. Arch. Intern. Med.* *9*, 219.

58. Konda, S., Noro, Y. Sawai, Y., and Tashiro, Y. (1962): A Role of Lymphocytes in Antibody Production; Antibody Formation Promoting Function of RNA Isolated From Lymphocytes, *Acta Haemat. Jap.* *25*, 159.

59. Kramer, M. and Straub, F. B. (1956): Role of Specific Nucleic Acid in Induced Enzyme Synthesis, Biochim. Biophys. Acta 21, 401.

60. Malmgren, R. A., Bennison, B. E., and Mc Kinley, T. W. (1952): The Effect of Guanazolo on Antibody Formation, J. Nat. Cancer Inst. 12, 807.

61. Hoyer, J. R., Hoyer, L. W., Good, R. A., and Condie, R. M. (1962): The Effect of 6-Mercaptopurine on Delayed Hypersensitivity in Guinea Pigs, J. Exp. Med. 116, 679.

62. Hitchings, G. H. and Ellion, G. B. (1963): Chemical Suppression of the Immune Response, Pharm. Rev. 15, 365.

63. Schwartz, R., Stack J., and Dameshek, W. (1958): Effect of 6 MP on Antibody Production, Proc. Soc. Exptl. Biol. and Med. 99, 164.

64. Schwartz, R., Eisner, R., and Dameshek, W. (1959): The Effect of 6 MP on Immune Responses, Clin. Res. 7, 39.

65. Schwartz, R., Eisner, R., and Dameshek, W. (1959): The Effect of 6 MP on Primary and Secondary Immune Responses, J. Clin. Invest. 38, 1394.

66. Dutton, R. W. (1960): The Inhibition of Antibody Formation in Vitro, Bioch. J. 74, 24.

67. La Plante, E.S., Condie, R. M., and Good, R. A. (1962): Prevention of Secondary Immune Response with 6-Mercaptopurine, J. Lab. Clin. Med. 59, 542.

68. Condie, R. M., Mennis, W., and Miller, C. (1961): Effect of 6 MP on Antibody Production, Fed. Proc. 20, 26.

69. Schwartz, R. and Dameshek, W. (1959): Drug Induced Immunological Tolerance, Nature 183, 1682.

70. Genghof, D. S., and Battisto, J. K. (1961): Antibody Production in Guinea Pigs Receiving 6-MP, Proc. Soc. Exptl. Biol. and Med. 107, 933.

71. Levin, R. H., Landy, M., and Frei, E. (1964): The Effect of 6-Mercaptopurine on Immune Response in Man, New Eng. J. Med. 271, 16.

72. Schwartz, R. (1962): Effect of 6 MP and Prednisone on Runt Disease, Blood 20, 114.

73. Dixon, F. J., Talmadge, D. W., and Maurer, P. H. (1952): Radiosensitive and Radioresistant Phases in Antibody Production, J. Immunol. 68, 693.

74. Taliaferro, W. H. (1957): Modification of the Immune Response by Radiation and Cortisone, *Ann. N.Y. Acad. Sci. 69*, 745.

75. Makinodan, T., and Gengozian, N. (1960): X-Ray Depression of the Recognition Mechanism of Antibody Forming Cells, in *Mechanisms of Antibody Formation*, M. Holub and L. Jarosková, eds. Pub. House of the Czechoslovak Academy of Sciences, Prague, p. 313.

76. Cole, L. J. and Alpen, E. L. (1962): Marrow Transplantation in X-Irradiated Dogs Treated with 6-MP and Urethane, in *Conf. on Bone Marrow Transplantation and Chemical Protection in Large Animals and Man*, V.A. Hospital, Long Beach, Calif.

77. Simic, M. M., Sljivic, V. S., and Petkovic, M. Z. (1961): Effects of Some Pyrimidine Analogues on the Formation of Circulating Antibody, *Bull. Inst. of Nuclear Sciences Boris Kidrich 11*, 235.

78. Flexner, J. B., Flexner, L. B., and Stellar, E. (1963): Memory in Mice as Affected by Intracerebral Puromycin, *Science 141*, 57.

79. Dixon, F. J., Bukantz, S. C., Dammin, G. J., and Talmadge, D. W. (1951): Fate of I^{131}-labelled Bovine Gamma-globulin in Rabbits, *Fed. Proc. 10*, 553.

80. Dixon, F. J., Maurer, P. H., and Deichmiller, M. P. (1954): Primary and Specific Anamnestic Antibody Responses of Rabbits to Heterologous Serum Protein Antigens, *J. Immunol. 72*, 179.

81. Speirs, R. (1964): How Cells Attack Antigens, *Sci. Amer. 210*, No. 2, 58.

82. Garvey, J. S., and Campbell, D. H. (1957): The Retention of S35-Labelled Bovine Serum Albumen in Normal and Immunized Rabbit Liver Tissue, *J. Exp. Med. 105*, 361.

83. Vredevoe, D. L., and Nelson, E. L. (1963): Induction of Anamnestic Response to BSA by Transfer of "Primed" Liver Cells, *Biochem. and Biophys. Res. Communications 10*, 221.

84. Rittenberg, M., and Nelson, E. L. (1960): Macrophages, Nucleic Acids, and the Induction of Antibody Formation, *Am. Nat. 94*, 321.

85. Ramón y Cajal, S. (1910): *Histologie du Système Nerveux*, Paris.

86. Sholl, D. A. (1956): *The Organization of the Cerebral Cortex*, Methuen, London.

87. Fischer, D. S. (1964): Theories of Antibody Formation: A Review, *Yale J. Biol. and Med. 37*, 1.

88. Breinl, F., and Haurowitz, F. (1930): Chemische Untersuchung des Präzipitates aus Hämoglobin und Anti-Hämoglobin-Serum unt Bemerkungen uber die Natur der Antikorper, Z. Physiol. Chemie, 192, 45.

89. Alexander, J. (1931): Some Intercellular Aspects of Life and Disease, Protoplasma 14, 296.

90. Mudd, S. (1932): A Hypothetical Mechanism of Antibody Formation, J. Immunol. 23, 423.

91. Pauling, L. (1940): A Theory of the Structure and Process of Formation of Antibodies, J. Amer. Chem. Soc. 62, 2643.

92. Burnet, F. M. (1941): The Production of Antibodies: A Review and Theoretical Discussion, Mc Millan and Co., Melbourne.

93. Bergmann, M. and Niemann, C. (1937): Newer Biological Aspects of Protein Chemistry, Science 86, 187.

94. Bergmann, M. and Niemann, C. (1937): On the Structure of Proteins, Cattle Hemoglobin, Egg Albumen, Cattle Fibrin and Gelatin, J. Biol. Chem. 118, 301.

95. Schweet, R. S., and Owen, R. D. (1957): Concepts of Protein Synthesis in Relation to Antibody Formation, J. Cell. Comp. Physiol. 50 (Suppl. 1), 199.

96. Jerne, N. K. (1955): The Natural Selection Theory of Antibody Formation, Nat. Acad. Sci. 41, 849.

97. Jacob, F. and Monod, J. (1961): Genetic Regulatory Mechanism in the Synthesis of Proteins, J. Molec. Biol. 3, 318.

98. Finch, L. R. (1964): Gamma-Globulin Operon: A Hypothesis for the Mechanism of the Specific Response in Antibody Synthesis, Nature 201, 1288.

99. Burnet, F. M., and Fenner, F. (1949): The Production of Antibodies, Second ed., Mc Millan and Co., Melbourne.

100. Burnet, F. M. (1957): A Modification of Jerne's Theory of Antibody Production, Using the Concept of Clonal Selection, Aust. J. Sci. 20, 67.

101. Burnet, F. M. (1960): Theories of Immunity, Perspect. Biol. and Med. 3, 447.

102. Burnet, F. M. (1959): The Clonal Selection Theory of Acquired Immunity, Vanderbilt Univ. Press, Nashville, Tenn.

103. Medawar, P. B. (1960): Theories of Immunological Tolerance, in *Ciba Foundation Symposium on Cellular Aspects of Immunity*, G. E. W. Wolstenholme and M. O'Connor, eds., Little Brown and Co., Boston, p. 134.

104. Lederberg, J. (1959): Genes and Antibodies, *Science* *129*, 1649.

105. Nossal, G. J., Makela, O., Engel, M. L., and Fefer, A. (1962): Cellular Proliferation in Immunity, *Stanford Med. Bull.* *20*, 32.

106. Baney, R. N., Vasquez, J.J., and Dixon, F. J. (1962): Cellular Proliferation in Relation to Antibody Synthesis, *Proc. Soc. Exp. Biol. Med.* *109*, 1.

107. Dutton, R. W. (1961): Importance of Cell Division for Antibody Production in an In Vitro System, *Nature* *192*, 462.

108. Nossal, G. J. V., and Lederberg, J. (1958): Antibody Production by Single Cells, *Nature* *181*, 1419.

109. Nossal, G. J. V. (1958): Antibody Production by Single Cells, *Brit. J. Exp. Path.* 39, 544.

110. Nossal, G. J. V. and Makela, O. (1962): Elaboration of Antibodies by Single Cells, *Ann. Rev. Microbiol.* 16, 53.

111. Nossal, G. J. V. (1962): Cellular Genetics of Immune Responses, *Adv. Immunol.* *2*, 163.

112. Coons, A. H. (1958): The Cytology of Antibody Formation, *J. Cell. Comp. Physiol.* *52* (*Suppl. 1*), 55.

113. White, R. G. (1958): Antibody Production by Single Cells, *Nature* *182*, 1383.

114. Attardi, G., Cohn, M., Horibata, K. and Lennox, E. S. (1964): Antibody Formation by Rabbit Lymph Node Cells, I, II, and III, *J. Immunol.* *92*, 335.

115. Cohn, M. and Lennox, E. S. (1960): Unpublished data cited in Discussion, p. 150 in *Ciba Symposium on Cellular Aspects of Immunity*, G. E. W. Wolstenholme and M. O'Connor, eds., Little Brown and Co., Boston.

116. Trentin, J. J. and Fahlberg, W. J. (1963): An Experimental Model for Studies of Immunologic Competence in Irradiated Mice Repopulated with "Clones" of Spleen Cells, in *Conceptual Advances in Immunology and Oncology*, M. D. Anderson Hospital Symposium, Hoeber and Co., New York, p. 66.

117. Lederberg, J. (1958): Genetic Approaches to Somatic Cell Variation: Summary Comment, *J. Cell. Comp. Physiol.* *52*, *Suppl. 1*, 383.

118. Owen, R. D. (1945): Immunogenic Consequences of Vascular Anastomoses Between Bovine Twins, Science 102, 400.

119. Anderson, D., Billingham, R. E., Lampkin, G. H., and Medawar, P. B. (1951): The Use of Skin Grafting to Distinguish Between Monozygotic and Dizygotic Twins in Cattle, Heredity 5, 379.

120. Billingham, R. E., Lampkin, G. H., Medawar, P. B., and Williams, H. L. (1952): Tolerance to Homografts, Twin Diagnosis, and the Freemartin Condition in Cattle, Heredity 6, 201.

121. Billingham, R. E., Brent, L., and Medawar, P. B. (1953): Actively Acquired Tolerance of Foreign Cells, Nature 172, 603.

122. Billingham, R. E., Brent, L., and Medawar, P. B. (1956): Quantitative Studies on Tissue Transplantation Immunity. III. Actively Acquired Tolerance, Phil. Trans. Roy. Soc. 239, 357.

123. Smith, R. T. (1961): Immunological Tolerance to Non-Living Antigens, Adv. Imm. 1, 67.

124. Hašek, M., Lengerová, A. and Hraba, T. (1961): Transplantation Immunity and Tolerance, Adv. Imm. 1, 1.

125. Hašek, M., Lengerová, A., and Vojtíšková, M., eds., (1961): Symposium on Mechanisms of Immunological Tolerance, Pub. House of the Czech. Acad. Sci., Praha.

126. Brent, L. and Medawar, P. B. (1958): Tolerance and Auto-Immune Phenomena, in Recent Progress in Microbiology, G. Tunevall, ed., Almqvist and Wilksells, Stockholm.

127. Dixon, F. J., and Maurer, P. H. (1955): Immunologic Unresponsiveness Induced by Protein Antigens, J. Exp. Med. 101, 245.

128. Battisto, J. R., and Miller, J. (1962): Immunological Unresponsiveness Produced in Adult Guinea Pigs by Parenteral Introduction of Minute Quantities of Hapten or Protein Antigen, Proc. Soc. Exptl. Biol. Med. 111, 111.

129. Sulzberger, M. B. (1929): Hypersensitiveness to Arsphenamine in Guinea Pigs. I. Experiments in Prevention and in Desensitization, Arch. Dermat. and Syph. 20, 669.

130. Chase, M. W. (1946): Inhibition of Experimental Drug Allergy by Prior Feeding of the Sensitizing Agent, Proc. Soc. Exptl. Biol. Med. 61, 257.

131. Chase, M. W. (1949): Interference with Induction of the Anaphylactic State by Prior Feeding of a Hapten-like Allergenic Chemical, Fed. Proc. 8, 402.

132. Battisto, J. R., and Chase, M.W. (1955): Immunologic Paralysis in Guinea Pigs Fed Allergenic Chemicals, Fed. Proc. 14, 456.

133. Felton, L. D. (1949): Significance of Antigen in Animal Tissues, J. Immunol. 61, 107.

134. Dresser, D. W. and Mitchison, N. A. (1960): The Cellular Basis for Immunological Memory, in Cellular Aspects of Immunity (Ciba Symposium), G.E.W. Wolstenholme and M. O'Connor, eds., Little Brown and Co., Boston.

135. Billingham, R. E. and Silvers, W. K. (1962): Some Factors that Determine the Ability of Cellular Inocula to Induce Tolerance of Tissue Homografts, J. Cell. Comp. Physiol. 60, 183.

136. Gombos, A., Tischler, V., Jacina, J. and Skokan, J. (1962): Successfully Transplanted Kidneys in Dogs, Ann. N.Y. Acad. Sci. 99, 787.

137. Mc Khann, C. F. (1962): Weak Histocompatibility Genes: The Effect of Dose and Pretreatment of Immunizing Cells, J. Immunol. 88, 500.

138. Guttmann, R. D. and Aust, J. B. (1961): Acquired Tolerance to Homografts Produced by Homologous Spleen Cell Injection in Adult Mice, Nature 192, 564.

139. Thorbecke, G. J., Siskind, G. W., and Goldberger, N. (1961): The Induction in Mice of Sensitization and Immunological Unresponsiveness by Neonatal Injection of BGG, J. Immunol. 87, 147.

140. Dresser, D. W. (1962): Specific Inhibition of Antibody Production, II. Paralysis Induced in Adult Mice by Small Quantities of Protein Antigen, Immunology, 5, 378.

141. Chutná, J. and Hraba, T. (1960): Attempt to Induce Immunological Tolerance in Rabbits by Antigen (HSA) - Antibody Precipitate, in Mechanisms of Immunological Tolerance, Hašek, M., Lengerová, A., and Vojtíšková, M., eds., Pub. House of the Czech. Acad. Sci., Praha, p. 95.

142. Smith, R. T. (1960): Studies on the Mechanism of Immunological Tolerance, in Mechanisms of Immunological Tolerance, Hašek, M., Lengerová, A., and Vojtíšková, M., eds., Pub. House of the Czech. Acad. Sci., Praha, p. 313.

143. Liacopolos, P., Halpern, B. N., and Perrament, F. (1962): Unresponsiveness to Unrelated Antigens Induced by Paralyzing Doses of BSA, Nature 195, 1112.

144. Konda, S., Noro, Y., and Sawai, Y. (1961): Influence of Orotic Acid on Antibody Formation, Naika Hokan 8, 62.

145. Mc Laren, A. (1961): Induction of Tolerance to Skin Homografts in Adult Mice Treated with 6-MP, *Transplant. Bull. 28*, 99.

146. Feldman, M., Globerson, A., and Nachtigel, D. (1960): The Reactivation of the Immune Response Following X-Irradiation and Drug Induced Immune Tolerance, in *Mechanisms of Immunological Tolerance*, Hašek, M., Lengerová, A., and Vcjtišková, M., eds., Pub. House of the Czech. Acad. Sci., Praha, p.305.

147. Šterzl, J. (1961): Study of Antibody Formation by the Use of Metabolic Inhibitors. I. The Effect of 6-Mercaptopurine on Specific Immune Reactions, *Folia Microbiol. 5*, 364.

148. Simonsen, M. (1962): Graft versus Host Reactions, Their Natural History, and Applicability as Tools of Research, *Progr. Allergy 6*, 349.

149. Miller, J. F. A. P. (1964): The Thymus and the Development of Immunologic Responsiveness, *Science 144*, 1544.

150. Chase, M. W. (1959): Immunological Tolerance, *Ann. Rev. Microbiol. 13*, 349.

151. Secarz, E. and Coons, A. H. (1959): Specific Inhibition of Antibody Formation During Immunological Paralysis and Unresponsiveness, *Nature 184*, 1080.

152. Kaplan, M. H., Coons, A. H., and Deane, H. W. (1950): Localization of Antigen in Tissue Cells. III. Cellular Distribution of Pneumococcal Polysaccharides Types II and III in the Mouse, *J. Exper. Med. 91*, 15.

153. Stark, O. K. (1955): Studies on Pneumococcal Polysaccharides. I. Biosynthesis of C^{14}-Labelled Type I Pneumococcal Polysaccharide, *J. Immunol. 74*, 130.

154. Argyris, B. F. (1963): Adoptive Tolerance; Transfer of the Tolerant State, *J. Immunol. 90*, 29.

155. Dietrich, F. M. and Weigle, W. O. (1964): Immunologic Unresponsiveness to Heterologous Serum Proteins in Adult Mice and Transfer of the Unresponsive State, *J. Immunol. 92*, 167.

156. Brooke, M. S. and Karnovsky, M. J. (1961): Immunological Paralysis and Adoptive Immunity, *J. Immunol. 87*, 205.

157. Smith, R. T. and Bridges, R. H. (1958): Immunological Unresponsiveness in Rabbits Produced by Neonatal Injection of Defined Antigens, *J. Exp. Med. 108*, 227.

158. Siskind, G. W. and Thorbecke, G. J. (1961): Immunological Unresponsiveness in Inbred Mice, *Fed. Proc. 20*, 27.

159. Dubert, J. M., and Paraf, A. (1957): Étude Quantitative des Conditions Nécessaires à l'Apparition d'un État de Tolérance Immunitaire, *Compt. Rend. Acad. Sci.* *244*, 686.

160. Humphrey, J. H. (1960): Discussion in *Mechanisms of Antibody Formation*, M. Holub and L. Jaroskova, eds., Pub. House of the Czech. Acad. Sci., Praha, p.353.

161. Garvey, J. S., Eitzman, D. V., and Smith, R. T. (1960): The Distribution of S^{35}-Labelled Bovine Serum Albumen in Newborn and Immunologically Tolerant Adult Rabbits, *J. Exp. Med.* *112*, 533.

162. Nossal, G. J. V., Fefer, A., and Mäkelä, O. (1960): Tolerance and Irradiation, in *Mechanisms of Immunological Tolerance*, Hasek, M., Lengerova, A., and Vojtiskova, M., eds., Pub. House of the Czech/ Acad. Sci., Praha, p.151.

163. Nossal, G. J. V. and Makela, O. (1961): Genetic Aspects of Antibody Formation, *Lab. Invest.* *10*, 1094.

164. Mäkelä, O. and Nossal, G. J. V. (1962): Accelerated Breakdown of Immunological Tolerance Following Whole Body Irradiation, *J. Immunol.* *88*, 613.

165. Stone, W. H. and Owen, R. D. (1963): The Loss of Partial Tolerance Following Sub-Lethal Irradiation, *Transplantation* *1*, 107.

166. Dixon, F. J. and Mc Conahey, P. J. (1963): Enhancement of Antibody Formation by Whole Body X-Irradiation, *J. Exp. Med.* *117*, 833.

167. Taliaferro, W. H. and Jaroslow, B. N. (1960): The Restoration of Hemolysin Formation in X-Rayed Rabbits by Nucleic Acid Derivatives and Antagonists of Nucleic Acid Synthesis, *J. Infect. Dis.* 107, 341.

168. Miller, J. F. A. P. (1962): Immunological Significance of the Thymus in the Adult Mouse, *Nature* *195*, 1318.

169. Miller, J. F. A. P., Doak, S. M. A., and Cross, A. M. (1963): Role of the Thymus in Recovery of the Immune Mechanism in the Irradiated Adult Mouse, *Proc. Soc. Exptl. Biol. Med.* *112*, 785.

170. Cross, A. M., Leuchars, E., and Miller, J. F. A. P. (1964): Studies on the Recovery of the Immune Response in Irradiated Mice Thymectomized in Adult Life, *J. Exptl. Med.* *119*, 837.

171. Claman, H. N. and Talmage, D. W. (1963): Thymectomy: Prolongation of Immunological Tolerance in the Adult Mouse, *Science* *141*, 1193.

172. Gorman, J. G. and Chandler, J. G. (1964): Struggle of Immunologically Competent and Incompetent Cells as a Theory of Tolerance, *Blood* *23*, 117.

173. Kartasheva, V. N., Vakarina, E. F., and Bodisko, V. P. (1960): Histochemical Study of Morphological Aspects of Immunity, *Trudy Moskov. Nauk Issledovatel. Inst. Vaktsin i Syvorotok 15*, 22.

174. Kartasheva, V. N., Bodisko, V. P., and Gorbunova, L. A. (1959): Concentration of Nucleic Acids in Cells of the Reticuloendothelial System in White Rats Immunized with Gaiskii Tularemia Vaccine, *Trudy Moskov. Nauk Issledovatel. Inst. Vaksin i Syvorotok 13*, 177.

175. Cottier, H., Odartchenko, N., Keisert, G., Hess, M.& Stoner, R. D. (1964): Incorporation of Tritiated Nucleosides and Amino Acids into Lymphoid and Plasmacytoid Cells during Secondary Response to Tetanus Toxoid in Mice, *Ann. N.Y. Acad. Sci. 113*, 612.

176. Cinader, B. and Dubert, J. M. (1955): Acquired Immune Tolerance to Human Albumen and the Response to Subsequent Injections of Diazo Human Albumen, *Brit. J. Exptl. Path. 36*, 515.

177. Harrison, T. H. et al, *Principles of Internal Medicine*, Fourth edition (1962), Blakiston, New York.

178. Stecher, P. G. ed. (1960): *The Merck Index of Chemicals and Drugs*, Seventh Edition, Merck, Rahway, New Jersey.

179. Taliaferro, W. H. and Taliaferro, L. G. (1950): The Dynamics of Hemolysin Formation in Intact and Splenectomized Rabbits, *J. Inf. Dis. 87*, 37.

180. Schmidt, G. and Thannhauser, S. J. (1945): A Method for the Determination of Desoxynucleic Acid and Ribonucleic Acid, and Phosphoproteins in Animal Tissues, *J. Biol. Chem. 161*, 183.

181. Ogur, M. and Rosen, G. (1950): The Nucleic Acids of Plant Tissues. I. The Extraction and Estimation of Desoxypentose Nucleic Acid and Pentose Nucleic Acid, *Arch. Bioch. Biophys. 25*, 262.

182. Vischer, E., and Chargaff, E. (1948): The Composition of the Pentose Nucleic Acids of Yeast and Pancreas, *J. Biol. Chem. 176*, 715.

183. Wiener, A. S., (1951): Origin of Naturally Occurring Hemagglutinins and Hemolysins, A Review, *J. Immunol. 66*, 287.

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